THE CELL ENVELOPE OF BIFIDOBACTERIUM BIFIDUM VAR. PENNSYLVANICUS

EFFECTS OF ANTIBIOTICS AND NUTRIENT DEPLETION

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GEORGE CONSTAND MOLENKAMP
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druk: Krips Repro Meppel

Promotor : Dr. J.H. Veerkamp

Co-Promotor: Prof.Dr. S.L. Bonting

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C O N T E N T S

The cell envelope of bacteria enables the organism to survive an exceptionally wide range of environments. It protects against mechanical damage, but allows the maintenance of a high intracellular concentration of salts and metabolites without osmotic rupture. The envelope determines also the shape of the organism. In Gram-positive bacteria the cell envelope is composed of two components: cell wall and cytoplasmic membrane. Although they have different structures and functions, both are prerequisites for a normal growth and functioning of the organism. The cellular localization of the cell wall and cytoplasmic membrane and the occurrence in the cytoplasmic membrane of certain enzymes and precursors, which are involved in the biosynthesis of cell wall, suggest a close interrelation between these envelope components. There is however relatively little known about the interactions between cell wall and cytoplasmic membrane in Gram-positive bacteria. We were interested in the nature of the interactions between the processes of cell wall and cytoplasmic membrane formation. For a study of this problem the Gram-positive organism: *Bifidobacterium bifidum* var. *pennsylvanicus* was used as a model, because in preceding investigations in our laboratory much information on the components of cell wall and cytoplasmic membrane of this organism was obtained. We designed experiments in which either the formation of cell wall or the formation of cytoplasmic membrane would be disturbed directly. If there exist tight interactions between formation of cell wall and of cytoplasmic membrane, then disturbances in one of these systems should be reflected by changes in the other system. To disturb the formation of the cell wall and cytoplasmic membrane we made use of certain antibiotics and growth inhibitory conditions. In the first place inhibitors of protein biosynthesis were applied. When protein biosynthesis is inhibited, the formation of cytoplasmic membrane protein, which is about 70% of the membrane, will be also inhibited. This means that one of the most direct consequences is distortion of the membrane formation. The question we posed ourselves was: what is the extent of the influence on the cell wall formation? In the second place we applied certain inhibitors of cell wall peptidoglycan synthesis, acting

at different levels in the cellular mechanisms. The question here was what consequences inhibition of cell wall biosynthesis would have on the membrane formation. In the third place some nonspecific growth inhibitory conditions for the organisms were used to study possibly interesting interactions between cell wall and cell membrane formations.

In Chapter 1 we shall discuss in general the cytoplasmic membrane of bacteria (1.1), the cell wall of Gram-positive bacteria (1.2) and the characteristics of the organism *B. bifidum* var. *pennsylvanicus* (1.3). In Chapter 2 a technique is described in which cell wall and cytoplasmic membrane formation is followed by measuring the incorporation of certain labeled precursors. The results obtained for normal and inhibited cells of *B. bifidum* var. *pennsylvanicus* are described. In Chapter 3 the metabolic stability of cell wall and cell membrane is studied in normal and inhibited labeled cells. Some interesting aspects of this study were further analyzed. Chapter 4 describes some important chemical and functional changes of the cytoplasmic membrane after growth inhibition which comprise: fatty acid composition, lipid composition, protoplast stability, sodium and potassium content of the cells, Rb^+-permeability and protein composition of the membranes. Chapter 5 comprises an electron microscopic study of the ultrastructure of normal and inhibited cells as a supplement to the chemical data obtained in the preceding chapters.

CHAPTER 1

THE CELL ENVELOPE OF GRAM-POSITIVE BACTERIA

1.1. THE CYTOPLASMIC MEMBRANE

1.1.1. Introduction

In this section we shall discuss the general features of the cytoplasmic membrane of Gram-positive organisms. Certain membrane properties have been investigated well in Gram-negative organisms, while this is not the case for Gram-positive organisms. Therefore the Gram-negative organisms are sometimes included in the discussion.

1.1.2. Localization in the cell

A schematic representation of the organization of the cell envelope of Gram-positive organisms is given in Fig. 1.

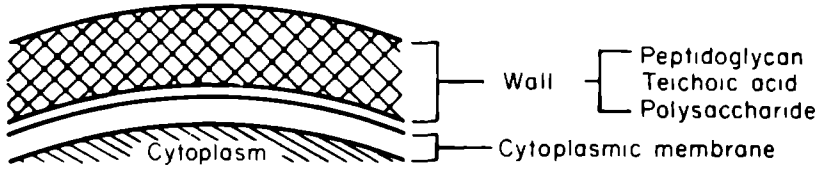

Fig. 1. The cell envelope of Gram-positive bacteria

The cell wall is situated at the outside of the cell. It is a rigid structure with a thickness of about 200-600 Å. Underlying the cell wall is the cytoplasmic membrane. The cytoplasmic membrane bounds the bacterial cytoplasm and is about 75 Å thick. Cytoplasmic membrane and cell wall have a fairly close contact, except in regions containing membrane intrusions or pockets of membrane vesicles. The structure

and function of cytoplasmic membranes was extensively reviewed by several authors (Korn, 1968; Rogers and Perkins, 1968; Op den Kamp et al., 1969; Salton, 1971; Machtiger and Fox, 1973).

1.1.3. Chemical composition

The cytoplasmic membrane of Gram-positive bacteria is a lipoprotein structure, formed by association of lipids and proteins by hydrophobic and hydrophilic interactions (Reaveley and Burge, 1972; Op den Kamp et al., 1969). The ratio of lipid to protein is about 1:3. In addition to lipids and proteins also ribonucleic acids and carbohydrates may be present. Table I presents the chemical composition of cytoplasmic membranes isolated from various Gram-positive bacteria.
The cytoplasmic membranes of most Gram-positive bacteria contain teichoic acids (Baddiley, 1968; Baddiley, 1972). In general the membrane teichoic acids are all polymers of glycerolphosphate in which the glycerol residues are linked through phosphodiester groups at the C-1 and C-3 position. The C-2 position of the glycerol residues carries glycosyl- or D-alanyl-substituents.

The predominant elelements of the cytoplasmic membrane of Gram-positive bacteria are structural and functional proteins. Many membrane-bound enzymatic activities have been detected in the cytoplasmic membranes of Gram-positive bacteria. Among them are glycolytic enzymes, Ca^{2+}- or Mg^{2+}-activated ATP-ase, cytochromes, autolysin, phospholipase C and enzymes involved in the biosynthesis of the cell wall (Machtiger and Fox, 1973).

The lipid content of the cytoplasmic membrane of Gram-positive organisms varies between 8 and 30%. The lipids and lipid-soluble components of the bacterial cell are located predominantly in the membrane system. The lipid composition of bacteria has been summarized by several authors (Kates, 1964; Asselineau, 1970; Lennarz, 1970; Goldfine, 1972). Phospholipids constitute the major fractions of the membrane lipids and have an essential structural function. Phosphatidylglycerol and its derivatives, especially diphosphatidylglycerol, often constitute

TABLE I. THE CHEMICAL COMPOSITION OF MEMBRANES ISOLATED FROM GRAM-POSITIVE BACTERIA
(in percents of total dry weight)

Organism	Protein	RNA	Lipid	Hexoses*	Nature of hexoses*
Bacillus subtilis	63	20	16	+	glucose
Bacillus licheniformis	75	0.8	28	+	glucose, galactose
Bacillus stearothermophilus	74	11	18	+	glucose
Bacillus megaterium	75	12	7	1.5	glucose
Bacillus megaterium M	67	1.3	19	4.8	glucose
Bacillus megaterium KM	65	5.1	20	8.0	glucose
Micrococcus lysodeikticus	68	2.3	23	++	glucose, galactose, mannose
Sarcina lutea	57	5.4	23	+	glucose, galactose, mannose
Staphylococcus aureus H	67	4.6	23	0.9	glucose
Streptococcus faecalis	46	2.7	32	+	glucose, galactose
Streptococcus group A	68	2.0	25	2.1	glucose
Streptococcus pyogenes	68	-	15	1.7	glucose
B. bifidum var. pennsylvanicus	70	8.3	8	12	glucose, galactose

* Present in glycolipids, teichoic acids and/or polysaccharide.

a high percentage of the phospholipids. The cytoplasmic membrane may also contain in addition to phospholipids glycolipids, mostly glycosyldiglycerides making up 5-40% of the total lipid content (Shaw, 1970; Sastry, 1974). Characteristic for the lipid composition of the cytoplasmic membrane in bacteria is the absence of sterols. An exception is formed by some Mycoplasma species which lack a normal cell wall and which are surrounded by a membrane only. These organisms are parasitic and may incorporate cholesterol from an extracellular source into their membrane lipids (Smith, 1964). However, in the lipids of the cytoplasmic membranes of bacteria, polyisoprenoids may be present (Lennarz and Scher, 1972). As we shall describe in the next section these polyisoprenoids play an important role in the biosynthesis of the cell wall. Phosphatidylcholine and sphingolipids are only present in some Gram-negative organisms. The apolar constituents of the lipids are mainly formed by fatty acyl groups. The Gram-positive organisms contain generally a large proportion of straight and branched fatty acids and a relatively low content of unsaturated fatty acids. Cyclopropane fatty acids can be present (Pohl and Wagner, 1972), whereas polyunsaturated fatty acids are mostly absent.

1.1.4. Function

The cytoplasmic membrane in the organism serves several functions. Much of the cellular organization in the Gram-positive organism is based upon the cytoplasmic membrane. Many enzymatic activities in the bacterial cell are associated with the cytoplasmic membrane, as we have indicated before. The cytoplasmic membrane contains vital components of the systems for electron transport, lipid synthesis of cell wall and also for permeation. The cytoplasmic membrane constitutes the main osmotic barrier of the cell. It is likely that the proper distribution of the chromosomes in the cytoplasmic membrane is involved in cell division and the proper distribution of the chromosomes in the daughter cells, although full evidence on this subject is still lacking. Besides having these functions the cytoplasmic membrane can constitute a place for the attachment of a flagella. Some of the most important functions of the cytoplasmic

membranes of Gram-positive bacteria are summarized in Table II.

TABLE II. FUNCTIONS OF THE CYTOPLASMIC MEMBRANE IN GRAM-POSITIVE BACTERIA

Active transport
Electron transport and oxydative phosphorylation
Secretion of extracellular protein toxins and enzymes
Cell wall synthesis
Protein synthesis and membrane-associated ribosomes
Phospholipid synthesis
Chromosome (DNA) anchoring, replication, and mitotic division

1.1.5. Mesosomes

In Gram-positive organisms there often occur internal membranous structures called "mesosomes" or "chondroids". A mesosome is formed by invagination of the cytoplasmic membrane. In thin sections mesosomes appear as pockets of cytoplasm-containing clusters of vesicles and tubuli, as membranous whorls, or as a combination of these structures. The phenomen of bacterial mesosomes is reviewed by Reusch and Burger (1973). Several functions have been proposed for the bacterial mesosome, but none of these are supported by convincing evidence. In the cases investigated, the dimensions of cytoplasmic membrane and mesosomal membrane were very similar, no significant differences existed between lipid and fatty acid composition of cytoplasmic and mesosomal membrane, but differences in protein composition may exist (Reusch and Burger, 1974). The mesosome is often found in contact with the bacterial chromatin, while in some organisms it is associated with the growing septum. Mesosomes may possibly have a function in cell division, but normal cell division has also been observed to take place in the absence of mesosomes.

The cytoplasmic membrane of bacterial cells, fixed by the Ryter and Kellenberger method (Ryter and Kellenberger, 1958) and studied by electron microscopy is visible as an alternately electron dense-light-dense layering, which is the characteristic profile for the unit-membrane postulated by Robertson (1959). The concept of the unit-membrane is an extension of the Davson-Danielli model (Davson-Danielli, 1935) for the general structure of plasma membranes. It can be summarized in three points:

(i) The membrane structure represents a principle, basically valid for all biological membranes.

(ii) The main structural element of the membrane consists of a continuous bilayer of phospholipids with varying amounts of glycolipids and neutral lipids, whereby the polar or hydrophilic parts of the lipids are arranged on both surfaces of the bilayer.

(iii) The lipid bilayer carries on both sides membrane proteins, which are arranged in extended (β-sheet) conformation, and held in position by ionic bonds.

More recent information, gained from the study of membrane proteins, seems to be in conflict with the postulate of the unit-membrane. An alternative view has been developed that considers the membranes to be aggregates of lipoprotein subunits (Benson, 1967). In the Benson model a predominant role is played by the hydrophobic linkage of the parafinic chains of the lipids to certain sites on the protein moiety. The polar headgroups of the phospholipids are not covered by proteins, but the possibility of electrostatic interactions between proteins and lipids cannot be excluded. However, it is important to note that neither the Robertson unit-membrane concept nor the Benson lipoprotein model has been proven (Korn, 1968). Korn (1968) also pointed out that no conclusion about the molecular architecture should be drawn from the profile of a membrane as seen in a thin section of a fixed and stained cell, studied by electronmicroscopy. There is still at this moment no certainty about the molecular architecture

of the bacterial cytoplasmic membrane and the plasma membrane of other organisms or cell types.

An alternative model for membrane structure was proposed by Singer and Nicholson (1972) (Fig. 2). They examined in detail several models of the gross structural organization of membranes in terms of thermodynamics of macromolecules and in the light of experimental evidence.

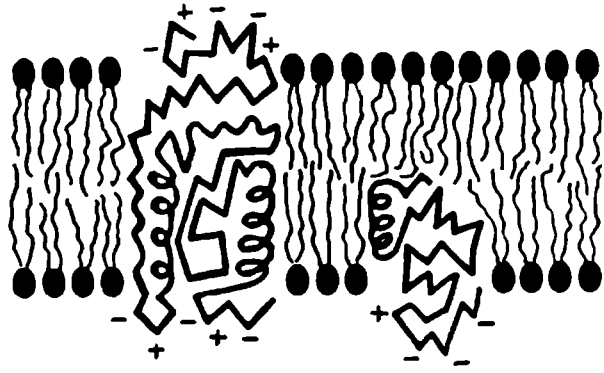

Fig. 2. Model for membrane structure. Phospholipids are arranged in a bilayer with the polar heads directed to the aqueous phase. The membrane proteins are shown as globular molecules partially embedded in and partially protruding from the membrane (Singer and Nicholson, 1972).

From this analysis it was concluded that a mosaic structure of alternating globular proteins and phospholipids bilayers was the only membrane model consistent with thermodynamic restrictions and with all experimental data. The mosaic model appears to be a fluid or dynamic structure. The proteins that are integral to the membrane are a heterogeneous set of globular molecules, each arranged in amphipathic structure. The ionic and highly polar groups protrude from the membrane into the aqueous phase and the apolar groups are largely buried

in the hydrophobic interior of the membrane. These globular molecules are partly embedded in a matrix of phospholipids. The bulk of the phospholipids is organized as a discontinuous fluid bilayer although a small fraction of the lipid may interact specifically with the membrane proteins. The fluid mosaic structure can therefore be considered as a two-dimensional solution of proteins in the viscous phospholipid bilayer solvent. The existence of lipid bilayer regions in membranes has been supported by X-ray diffraction data for membranes of *Mycoplasma* (Engelman, 1970). Further evidence for the presence of lipid bilayer regions came from the comparative study of nitroxide spin labels in lipids and membrane (Hubble and McConnell, 1969; Libertini et al., 1969) and from the study of temperature-induced phase transitions in both lipids and membranes by differential calorimetry (Stein et al., 1969).

1.1.7. Membrane function and lipid composition

The lipid composition of the bacterial cytoplasmic membrane can have marked physical consequences, which may affect the membrane function. Since the apolar part of phospholipids appears to play an important role on the functioning of many membrane-bound enzymes, the nature of the apolar part of the molecules may be an important factor, in controlling their activity (Tourcans and Jain, 1974). The membrane lipids can undergo reversible thermotropic transitions which may be detected by differential scanning calorimetry and X-ray diffraction techniques. It is suggested that these transitions are related to liquid crystalline-fluid transitions of the lipid phase which can be qualitatively expressed as "melting" of the fatty acid chains within a bilayer. The transition temperature can be varied by changing the fatty acid composition of the membrane lipids. Factors which favor a fluid rather than a crystalline nature are: (i) the extent of unsaturation of the fatty acids; (ii) shortening of chain length; (iii) cyclopropane grouping. At the growth temperature the fatty acids in the cytoplasmic membrane are in a liquid state. This provides an environment in which many membrane-bound enzymatic reactions take place. For certain bacterial organisms (*Escherichia coli, Mycoplasma*) it

has been demonstrated that the nature of the fatty acids in the cytoplasmic membrane affects the functioning of membrane-bound enzymes and of cell membrane permeability. Bacterial mutants of *E. coli*, unable to synthesize unsaturated fatty acids, need these substances as a growth factor (Sibert and Vagelos, 1967). In the absence of unsaturated fatty acids in the culture medium cell death occurs. This may result from disturbances of membrane functions, caused by alterations in the physical state of the lipids. The changes are in overall membrane permeability and probably in membrane-bound enzymatic activities (Cronan and Vagelos, 1972).

The fatty acid composition of unsaturated fatty acid auxotrophs of *E. coli* and of *Mycoplasma* organisms can be influenced within certain limits by the fatty acid composition of the growth medium of the micro-organisms (Schairer and Overath, 1969; McElhaney and Tourtelotte, 1969). In an unsaturated fatty acid auxotroph of *E. coli* the fatty acid composition of the bacterial cytoplasmic membrane influenced the transport rates of β-glycosides and β-galactosides (Wilson and Fox, 1971). The Arrhenius plots were biphasic and showed characteristic transition points. The transition temperatures for transport, mediated for single systems, increased with an increasing degree of saturation of the fatty acids. The transition points obtained for the independent β-glucoside and β-galactoside transport were identical, indicating that the transition point temperature for the transport-activities are a property of the bulk membrane lipids rather than a consequence of specific lipid-protein interactions. The Arrhenius plots for membrane-bound Mg^{2+}-dependent ATPase in *Mycoplasma laidlawii* are also biphasic with characteristic transition point temperatures (De Kruyff et al., 1973). When the fatty acid composition of the membrane lipids was varied by changing the fatty acid composition of the culture medium, different transition points in the Arrhenius plots occurred. A correlation was found with the transition temperature of the lipid phase as measured by differential scanning calorimetry. The fatty acid composition of the bacterial membrane lipids does not only influence the membrane-bound enzymes but can also be important for the effective activity of bactericides.

This was shown with an unsaturated fatty acid mutant of *E. coli* grown on structurally diverse unsaturated fatty acids. The temperature profile for Colicin k action on this organisms was influenced by the fatty acid compostion of the membrane phospholipids (Plate, 1973).

Another form of a possible interaction between enzymes and lipids may exist by the dependence of some membrane enzymes on the presence of lipid activators. An example is given by the isoprenoid-alcohol phosphokinase (Higashi and Strominger, 1970), and the phospho-N-acetyl-muramic acid pentapeptide translocase (Umbreit and Strominger, 1972). Both enzymes are activated by phospholipids.

1.1.8. Membrane permeability

An important membrane function is the regulation of the permeability of the microbial cell. There are several studies which indicate a possible correlation between fatty acid composition and permeability (De Gier, 1968; Scarpa et al., 1971; Haest et al., 1972; McElhaney et al., 1973; Van der Neut-Kok et al., 1974). In liposomes of synthetic lecithins with variable chain length the permeability of non-electrolytes like glycerol and erythritol was enhanced by introduction of fatty acids with double bonds or short chain length (De Gier, 1968). Scarpa et al. (1971) showed an increasing permeability for K^+ ions with increasing unsaturation of the fatty acids. These observations are not only restricted to artificial membranes. Intact cells of *Mycoplasma laidlawii* with different fatty acid composition, as well as liposomes derived from the lipids of these cells, showed an increased non-electrolyte permeability when branched, unsaturated or short-chain fatty acids were incorporated (McElhaney et al., 1973). Van der Neut-Kok et al. (1974) have reported that the valinomycin-induced permeability of *Mycoplasma laidlawii* for Rb^+ and K^+ ions is increased with higher unsaturation of the membrane lipids. Efflux of K^+ decreased at lower temperatures and became zero below the temperature of the gel-liquid crystalline phase transition of the membrane lipid. It was suggested that the membrane "fluidity", which

is determined by the nature of the fatty acid chains in the membrane lipids, is involved in membrane permeability (Van der Neut-Kok et al., 1974).

1.1.9. Membrane synthesis

At a morphological level synthesis of the cell membrane involves: (i) continuous extension of peripheral membrane between two divisions; (ii) formation of cross-wall membrane and septation of DNA-membrane attachment sites. Most studies have been confined to the location of the sites of synthesis. Membrane synthesis may occur at many disperse foci or in an equatorial band. The latter possibility, with its corollary that the old membrane components are conserved, is the basis of the replicon theory of genome segregation (Jacob et al., 1963).

The topology of membrane growth has been reviewed by Kepes and Autissier (1972). Investigations on *B. subtilis* (Mindich and Dales, 1972) and on *E. coli* (Tsukagoshi et al., 1971; Overath et al., 1971) indicate a randomly dispersed incorporation of lipids into the bacterial membrane. The results with *B. megaterium* KM (Morrison and Horowitz, 1970) and *B. cereus* (Fitz-James and Young, 1969) are contradictory and show a localized lipid incorporation into the membrane. However, Bacon and White (1974) could not demonstrate the existence of localized membrane synthesis in *B. megaterium* during sporulation. In connection with these observations it is important to take note of the fact that the thermal mobility of the lipids, their turnover and their chemical heterogenity might obscure a specific focus in the incorporation of membrane lipids.

Studies, in which segregation of certain inducible characteristics were followed, indicate a conservation of membrane proteins in *E. coli* (Autissier and Kepes, 1971), implying localized insertion of membrane protein. However, studies on segregation of preformed and newly formed membrane proteins into cells and minicells of *E. coli* (Wilson and Fox, 1971) did not support models of membrane assembly involving

localized membrane growth at the poles or at an equatorially site with conservation of the structure of old and new membranes.

Very few studies of membrane synthesis in relation to cell age have been made. Discontinuous synthesis of phospholipids during the cell cycle of *B. megaterium* has been claimed (Daniels, 1969). Ohki (1972) has demonstrated discontinuous increases in cytochrome b_1 synthesis and glycerophosphate transport with a simultaneous increase in turnover of phosphatidylglycerol. Investigations of Sargent (1973) on the synthesis of the cytoplasmic membrane of *B. subtilis* indicated that during the cell cycle certain proteins are added to the membrane from the cytoplasm and that there is a period of zero net synthesis in which there is an efflux of proteins from the membrane. In contrast to the results on *B. megaterium* (Daniels, 1969) phospholipid synthesis appeared to be continuous throughout the cell cycle.

1.1.10. Co-ordination of membrane protein and -lipid synthesis

A number of investigations concerning the co-ordination of protein and lipid synthesis in the bacterial membrane have been carried out. In *E. coli* the stringent coupling of ribonucleic acid synthesis and protein synthesis is under genetic control. Mutants have been isolated in which this control is relaxed. Sokawa et al. (1968) showed with amino acid auxotrophs of *E. coli* that lipid synthesis is similarly coupled to protein synthesis, and that in relaxed mutants this control is also relaxed. The interrelation between protein and lipid synthesis appeared to be under the same genetic control as that between ribonucleic acid and protein synthesis. In this control the component guanosinetetraphosphate seems to play an important role. A strict correlation between guanosinetetraphosphate accumulation and inhibition of phospholipid synthesis has been demonstrated (Merlie and Pizer, 1973). Guanosinetetraphosphate is directly involved in the regulation of enzymic steps, specific for the synthesis of phospholipids (Merlie and Pizer, 1973).

Kahane and Razin (1969) studied membrane protein and membrane lipid synthesis in *M. laidlawii*. After inhibition of protein synthesis in the membrane the synthesis of lipids continued, resulting in the formation of membranes with an increased lipid content and a lower density as compared to the normal membranes. Withholding of glycerol from a *B. subtilis* glycerol auxotroph caused an immediate stop of the net phospholipid synthesis (Mindich, 1970). In the absence of net lipid synthesis the incorporation of amino acids into the membrane and the increase of membrane protein continued. Membranes isolated from glycerol-deprived cells exhibited a greater buoyant density than those from normal cells, indicating a higher protein content. In this organism the inhibition of protein synthesis resulted also in membranes having a higher lipid content than that of normal cells. Glaser (1973) found that in a temperature-sensitive mutant, defective in phospholipid synthesis at the restrictive temperature the net phospholipid synthesis was stopped, but protein synthesis as well as RNA synthesis and DNA synthesis also ceased. In an oleate auxotroph of *E coli* K-12 oleate starvation caused cessation of the synthesis of protein, ribonucleic acid, desoxyribonucleic acid and phospholipid (Henning et al., 1969). During glycerol deprivation in a glycerol auxotroph of *S. aureus* there was a cessation of net phospholipid synthesis, but under these conditions the synthesis of protoheme, heme and non-specific membrane protein continued at the predeprivation rate (Ray et al., 1973). In a mutant of *E. coli*, defective in membrane phospholipid synthesis, a net phospholipid syntheis was not required for a continued protein synthesis (Bell, 1974). It was also found that decreasing the lipid synthesis in *E. coli* by 90% through inhibition of its β-ketoacyl-thioesterase activity with cerulenin, did not affect protein synthesis (Goldberg et al., 1973).

The majority of the reports on the interdependence of membrane protein and membrane lipid synthesis suggest that there is no tight coupling between these two processes and that the incorporation of protein and lipid into the membrane are mutually independent.

1.2. THE CELL WALL OF GRAM-POSITIVE BACTERIA

1.2.1. Introduction

Studies on bacterial cell walls have been summarized in reviews of Salton (1964), Ghuysen et al. (1968), Osborn (1969), Rogers (1970), Braun and Hantke (1974). In this section various aspects of structure and biosynthesis of the peptidoglycan of Gram-positive bacteria, and where necessary of Gram-negative bacteria, will be discussed.

1.2.2. Cellular localization and function

The bacterial cell wall is a rigid, water-insoluble evelope, surrounding the cytoplasmic membrane. This supporting structure, of which the thickness ranges from 200-600 Å in general, is essential for maintaining the cell alive under normal environmental conditions, which are often characterized by hypotonicity. Besides protecting the cell against osmotic forces, the cell wall maintains the shape of the organism. The cell wall is necessarily involved in an important way in the processes of growth and division. Although the cell wall is a rigid structure with considerable mechanical strength, it is nevertheless freely permeable for cellular products and nutrients. Under certain conditions even large molecules with a molecular weight of up to 120 000 (Scherrer and Gerhardt, 1971) can penetrate the wall. The cell wall components constitute a substantial proportion of the metabolic products of the cell. The cell wall frequently comprises up to 25% of dry weight. It is often the site of important antigenic material, and in many cases the group- or type-specific antigens are localized in the outer structures of the cell (McCarty and Morse, 1965).

1.2.3. Chemical composition

The peptidoglycan of the cell wall of Gram-positive bacteria is mainly responsible for its mechanical strength (Anderson et al., 1966). The peptidoglycan was formerly also designed as "mucopeptide" (Mandel-

stam and Rogers, 1959), "glucosaminepeptide" (Salton, 1964), or "murein" (Weidel and Pelzer, 1964). Peptidoglycan is present in nearly all Gram-positive bacteria and constitutes 50-90% of the cell wall. Other cell wall components comprise: proteins, teichoic acids, and polysaccharides. None of these has been isolated as a distinct anatomical structure and all are probably bound to the peptidoglycan by covalent linkages. As a consequence, these components are released when the integrity of the peptidoglycan structure is lost. The non-peptidoglycan components of the bacterial cell wall are, due to their external location on the cell surface, the sites of important biological activities. They may carry antigenic specificities and be specific receptors for phage fixation or endotoxins. We shall shortly mention some features of the non-peptidoglycan components of the cell wall and then focus the discussion further on the structurally most important component: the peptidoglycan.

In the cell wall of Gram-positive organisms proteins can occur. Proteins in the bacterial cell wall comprise in most cases protein antigens. The most extensive information available on protein antigens of bacterial cell walls comes from studies of *Streptococci* (Lancefield, 1962; Hahn and Cole, 1963). The proteins, which carry M, R, and T antigens of groups *A*, *C*, and *G Streptococci*, are thought to be on the surface of the wall and can be removed by trypsine action without loss of viability. The M-protein of group *A Streptococci* has been identified as a virulence factor and is responsible for the production of type specific antibodies in man, which is the basis of type specific immunity.

Teichoic acids are important components of both cell wall and cell membrane. The teichoic acids of the cell wall resemble the membrane teichoic acids (see *1.2.3.*) but in addition to glycerophosphate polymers examples of polyribitolphosphates have been found in which phosphodiester linkages join the 1- and 5-position on adjacent ribitol residues. Glyceryl- and D-alanine ester residues can be present. In many bacteria, wall teichoic acids are attached to the mucopeptide through their terminal phosphate residues. They play an

important role as phage receptor site. In contrast to the membrane teichoic acids, the cell wall teichoic acids are not always present (Baddiley, 1972).

Gram-positive organisms may additionally contain considerable amounts of polysaccharides in their cell wall. An example is given by the *Streptococci*, where the polysaccharide consist of rhamnose in combination with either glucose, galactose, N-acetylglucosamine or N-acetylgalactosamine (Ghuysen et al., 1968).

1.2.4. Chemical structure of peptidoglycan

The cell wall peptidoglycan is a polymeric structure containing acylated aminosugars and three to six different amino acids. The basic structure is a heteropolymer, consisting of glycan strands cross-linked through short peptides. The glycan moiety of the peptidoglycan is remarkably uniform for all bacteria. It usually consists of alternating β-1,4-linked N-acetyl-glucosamine and N-acetylmuramic acid residues. The glycan reveals only few variations such as acetylation or phosphorylation of the 6-position in the muramyl groups (Ghuysen, 1968). Only glucomuramic acids and no galactomuramic acids occur in the wall (Wheat and Ghuysen, 1971). Concerning the length of the glycan chains only an average chain length can be given since the glycans are polydisperse. The average length varies in the different organisms between 10 and 65 disaccharide units. The length of a single disaccharide unit is 1.03 nm (Keleman and Rogers, 1971). However, one has to take into account that during isolation of cell wall the action of autolytic enzymes may reduce the average chain length. The possibility exists that the average chain length is larger in vivo than in preparations of cell wall. The peptide moiety of the peptidoglycan is bound through its N-terminal to the carboxyl group of muramic acid, and it contains alternating L- and D-amino acids. Fig. 3 shows the chemical structure of the peptidoglycan chain.

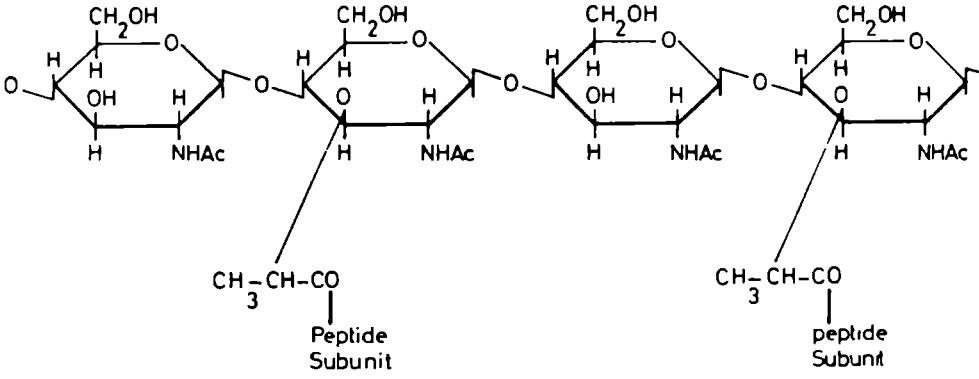

Fig. 3. Chemical structure of a part of the peptidoglycan chain

A typical feature of peptidoglycan is the occurrence of D-amino acids. Usually L-alanine is bound to the muramic acid, followed by D-glutamic acid. D-glutamic acid is linked by its γ-carboxyl group to an L-diamino acid and to this diamino acid (e.g. lysine, diaminopimelic acid, ornithine) D-alanine is attached. This part of the peptidoglycan peptide will be designed further as "pentapeptide subunit". Cross-linking between two peptidoglycan chains occurs by peptide bridges. These bridges may extend from the free ω-amino group in one pentapeptide subunit to the D-ala carboxyl group of the other pentapeptide subunit. The interpeptide bridges show great variations in their chemical structure for different species. The resulting peptidoglycan structure is a tight network, which is in fact an enormous macro-molecule encompassing the entire bacterium. The chemical composition and the structure of peptidoglycan of different taxonomical types have been reviewed by Schleifer and Kandler (1972).

1.2.5. Three-dimensional structure of peptidoglycan

Knowledge about the three-dimensional structure of peptidoglycan is generally lacking. An exception is the peptidoglycan of *E. coli*, of *Spirillum serpens* and of *Lactobacillus plantarum*, for which some quantitative data are known. Braun et al. (1973) found that the sur-

face area per repeating peptidoglycan unit for the cell envelope of *E. coli* is constant an amount of 1.3 nm^2. From this fact it was concluded that at most three peptidoglycan layers could be present, but a single layer is needed to construct a regular, covalently fixed peptidoglycan in two dimensions. The model for *E. coli* specifies a monomolecular layer in which the disaccharide units are each 1.03 nm long and the polysaccharide chains, all parallel, are 1.25 nm apart. The cross-linking peptide side chains have the same atomic co-ordinates and are arranged above or below the polysaccharide chains. Based on X-ray diffraction data, infrared spectra, density of peptidoglycan and on theoretical considerations, Formanek et al. (1974) proposed a model for the peptidoglycan structure of *S. serpens* and *L. plantarum*. The structure of the peptidoglycan chains is similar to that of chitin and cellulose. The peptides consisting of alternating D- and L-amino acids have a helical conformation. They are linked to the carbohydrate chains through their N-terminus and form two hydrogen bonds with the sugar residues. There is an angle of about 150^{o} between the carbohydrate and peptide chains.

1.2.6. The biosynthesis of peptidoglycan

The biosynthesis of cell wall peptidoglycan has been reviewed by several authors (Ghuysen, 1968; Osborn, 1969; Rogers, 1970; Braun and Hantke, 1974). The process of biosynthesis requires involvement of cytoplasmic enzymes as well as enzymes in intimate contact with the cytoplasmic membrane. Cell wall precursors are synthesized in the cell cytoplasm and then transferred to the membrane site involved in polymer formation. The cell wall peptidoglycan synthesis can be subdivided into three elementary stages: (i) Synthesis of nucleotide precursors (UDP-GlcNAc, UDP-MurNAc-pentapeptide) and of intermediates, involved in the cross-linking of peptidoglycan. This phase constitutes the cytoplasmic phase of the synthesis. (ii) Translocation of GlcNAc and phospho-MurNAc-pentapeptide and other intermediates to the cytoplasmic membrane, where membrane-associated enzymes catalyze the synthesis of nascent peptidoglycan (peptidoglycan in which peptide

cross-linking has not yet been realized). (iii) Cross-linking of nascent peptidoglycan by extracellular enzymes, resulting in an external network covering the bacterial surface. We shall discuss each of the three elementary stages in cell wall synthesis in some detail.

In the cytoplasm of the cell the nucleotide precursors are formed in the following sequence of reactions:

(1) GlcNAc-1-P + UTP $\rightarrow$ UDP-GlcNAc + PP_i

(2) UDP-GlcNAc + phosphoenolpyruvate $\rightarrow$ UDP-GlcNAc-pyruvate-enolether

(3) UDP-GlcNAc-pyruvate-enolether + NADPH + H^+ $\rightarrow$
UDP-GlcNAc-lactic-acid ether + $NADP^+$

The product of reaction (3) is identical with UDP-NAc-muramic acid (UDP-MurNAc). The reduction of UDP-GlcNAc-pyruvate-enolether is catalyzed by uridine-diphospho-N-acetyl-enol-pyruvyl glucosamine reductase. This enzyme is activated by a number of monovalent cations, especially K^+ (Taku and Anwar, 1973). Sequential incorporation of activated amino acids into the product of reaction (3), UDP-MurNAc, leads to the formation of the peptide subunit.

(4a) UDP-MurNAc + A_1 $\rightarrow$ UDP-MurNAc-A_1

(4b) UDP-MurNAc-A_1 + A_2 $\rightarrow$ UDP-MurNAc-A_1-A_2

(4c) UDP-MurNac-A_1-A_2 + A_3 $\rightarrow$ UDP-MurNAc-A_1-A_2-A_3

The enzymes involved in these reactions are dependent upon the presence of Mn^{2+} or Mg^{2+} ions (Neuhaus, 1962).
In a separate sequence of reactions D-alanyl-D-alanine is synthesized.

(5) L-ala $\rightarrow$ D-ala

(6) D-ala + D-ala + ATP $\rightarrow$ D-ala-D-ala + ADP + P_i

Reaction (5) is catalyzed by the cytoplasmic enzyme: alanine-racemase, which requires pyridoxalphosphate as a co-factor. D-alanyl-D-alanine is synthesized from two D-ala molecules (reaction (6)). This reaction is catalyzed by D-alanyl-D-alanine synthethase. The latter enzyme is inhibited by the reaction product (Neuhaus and Lynch, 1964). A necessary factor is the presence of Mg^{2+} and K^{+} ions. The D-alanyl-D-alanine is transferred to the UDP-MurNAc-tripeptide:

(7) UDP-MurNAc-tripeptide + D-ala-D-ala → UDP-MurNAc-tripeptide-D-ala-D-ala

The pentapeptide product is then transferred from the cytoplasmic uridylic acid carrier to a cytoplasmic membrane carrier which is a phospholipid. This translocation reaction is catalyzed by the enzyme N-acetyl-muramyl-pentapeptide translocase.

(8) UDP-MurNAc-pentapeptide + lipid-P → MurNAc(-pentapeptide)-P-P-lipid + UMP

In addition to requiring Mg^{2+} ions, the translocase is stimulated by monovalent cations as K^{+} and NH_4^{+} (Heydanek et al., 1970). The product of translocation contains a pyrophosphate link between the MurNAc-pentapeptide moiety and the phospholipid carrier molecule. The lipid carrier was identified by Higashi et al. (1967) as a C_{55}-isoprenoid alcohol phosphate, which contained 11 isoprene units, each having a double bond (undecaprenyl phosphate). In *S. aureus* the undecaprenyl phosphate contains two internal trans double bonds (Sweeley et al., 1970). It has been suggested that in addition to its role in peptidoglycan biosynthesis the undecaprenyl phosphate participates also in teichoic acid and capsular polysaccharide-biosynthesis in bacteria (Douglas and Baddiley, 1968; Brooks and Baddiley, 1969; Hussey and Baddiley, 1972).

In a subsequent reaction N-acetyl-glucosamine is attached to the moiety bound to the phospholipid carrier.

(9) MurNAc(-pentapeptide)-P-P-lipid + UDP-GlcNAc →
GlcNAc-MurNAc(-pentapeptide)-P-P-lipid + UDP

The last membrane-bound process is the incorporation of the amino acid(s) which will form the peptide cross-links in the completed peptidoglycan. The incorporation of these amino acid(s) may involve the participation of amino-acyl transfer RNA (Thorndike and Park, 1969).

(10) GlcNAc-MurNAc(-pentapeptide)-P-P-lipid + Az →
GlcNAc-MurNAc(-pentapeptide)-P-P-lipid
|
Az

The disaccharide-peptide moiety is then released from the membrane-bound phospholipid carrier and transferred to an acceptor outside the cytoplasmic membrane. This acceptor is probably a part of the peptidoglycan structure already present in the bacterial surface.

(11) GlcNAc-MurNAc(-pentapeptide)-P-P-lipid + Acceptor →
|
Az
Acceptor-GlcNAc-MurNAc-pentapeptide + lipid-P-P
|
Az

This reaction involves a transglycosilation. The phospholipid carrier, released after transfer of the disaccharidepeptide precursor to the acceptor site, is regenerated by the action of a pyrophosphatase (Siewert and Strominger, 1967).

(12) Lipid-P-P → Lipid-P + P_i

The phospholipid then participates in a new sequence of biosynthesis reactions. The last reaction, needed for completion of the peptidoglycan, is the closing of the cross-linking interpeptide bridges. This reaction is catalyzed by a transpeptidase and occurs in most cases by binding of the free end of the bridge to the fourth amino acid residue of a peptide unit of another glycan chain. The terminal D-alanine of the peptide unit is released in this process. No external source of energy, such as ATP, is required for the transpeptidation reaction.

The energy is provided by the concomitant release of D-alanine.

However, the exact way in which the lipid-P-bound precursors are polymerized into the preexisting peptidoglycan is still largely unknown. Concerning this question two possibilities are in discussion.

(i) the first step in the precursor polymerization is transpeptidation, followed by transglycosilation. This implies that the peptidoglycan chains are cross-linked to preexisting cell wall peptidoglycan before release from the membrane carrier occurs.

(ii) transglycosilation with release of the membrane bound carrier and attachment to preexisting peptidoglycan followed by transpeptidation.

Since no polymerized peptidoglycan has been found to be bound to the lipid carrier, it seems very probable that only the extracellular polymerized peptidoglycan is the acceptor for the lipid-linked precursors (Braun and Bosch, 1973). Mirelman et al. (1972, 1974) studied the biosynthesis of peptidoglycan in a cell-free system of *M. lysodeikticus*. They showed that penicillin not only inhibited the transpeptidation reaction but also the cross-linking of newly synthesized peptidoglycan in preexisting cell wall. An attachment of the UDP-MurNAc-precursor to the cell wall was only inhibited for about 70% by penicillin even at concentrations that completely block transpeptidation as measured by the release of D-alanine. This is probably caused by elongation of saccharide strands that occurs by transglycosilation. They concluded that cell wall elongation and growth is the result of a concerted operation by: (i) transpeptidation as a mechanism for attaching newly synthesized chains to free amino groups of the existing peptidoglycan and (ii) transglycosilation for the elongation of the glycan chains. Recently Ward and Perkins (1974) showed that in vitro incorporation of newly synthesized peptidoglycan chains can occur by cross-linking to the preexisting cell wall.

1.2.7. Expansion of the peptidoglycan structure during cell growth

Extension of the cell wall peptidoglycan network, which is a consequence of cell growth, implies that new molecules are inserted into

an already existing structure, in which most of the acceptor sites are already involved in covalent binding. Introduction of new molecules can only be realized if part of the potential acceptor sites is freed by clearing of existing bonds. The new material is bound by a transpeptidation reaction (Strominger et al., 1967). The cleavage of existing bonds is not well understood, but it should take place without damaging the cell wall and endangering the osmotic protection of the cell. Cleavage of peptidoglycan bonds is known to occur by enzymatic action. A number of bacterial species have been reported to contain autolytic enzymes which are able under appropriate conditions to cause hydrolysis of various susceptible bonds in the peptidoglycan. These autolytic enzymes comprise amidases as well as glycosidases (Ghuysen and Shockman, 1973). The presence of such enzymatic activities in rapidly growing and dividing bacterial cultures was suggestive for their potential role in cell growth and division. Higgins et al. (1970) showed that in *S. faecalis* both centripetal and peripheral wall extension resulted from cell wall synthetic activity near or at the leading edge of the septum. Cellular autolytic activity (β-N-acetylmuramide glycan hydrolase) appeared to be located in the same area. Thompson (1971) proposed a model for bacterial cell wall synthesis in which autolytic enzymes played an important role. Cleavage of preexisting peptidoglycan, to provide acceptor sites for peptidoglycan synthesis, was not a prerequisite for growth and expansion. Polymerization was proposed to occur on a membrane-bound lipid precursor. The strip of septum produced extends away from the membrane. Autolytic enzymes split it into two halves which grow towards opposite poles of the cell. The balance between synthesis and autolysis determines whether elongation or septum formation occurs.

There are, however, several indications that autolytic enzymes are not strictly necessary for cell wall growth. Mutants have been isolated which had a low activity (Fan and Beckman, 1972). Despite their low autolytic activity these mutants exhibited growth. Fan et al. (1972) were able to isolate a mutant which lacked at least two autolytic enzymes simultaneously at a nonpermissive temperature. As a result chain formation and bizarre shapes developed, but growth of the cell

wall continued. The mutant could be phenotypically reverted by the addition of lytic enzymes to the growth medium. This shows that in the absence of lytic enzymes expansion of the peptidoglycan network is still possible. Another indication in this direction is the report that in *B. subtilis* most muramic acids residues contain peptide bridges (Warth and Strominger, 1971). In *B. subtilis* one of the most important autolytic enzymes is: N-acetyl-muramic-acid-L-alanine amidase. Involvement of this enzyme in the process of cell wall enlargement would imply that many muramic acid residues lack peptide bridges, which had not been found. Despite the suggestive evidence for the role of autolysins in peptidoglycan synthesis, cell division, cell separation and remodeling of cell shape, no unequivocal evidence for an indispensable function in peptidoglycan synthesis has been reported (Braun and Hantke, 1974). An interesting alternative to the function of hydrolytic enzymes for growth of the cell wall was proposed by Fiedler and Glaser (1973). Transpeptidation reactions could occur between two peptidoglycan strands causing transfer of a strand from one binding site to another. This would result in movement of peptidoglycan strands with respect to each other and would allow expansion of the network. Mirelman et al. (1974) suggested also as one possible function of the cell wall endopeptidase to prepare more space for enlargement of the cell wall.

1.2.8. Sites for bacterial peptidoglycan synthesis

Theoretically the deposition of newly synthesized peptidoglycan can occur by random insertion in distinct growth zones. In rod-shaped organisms the process can be differentiated in two components: formation of the septum and deposition of lateral cell wall leading to elongation of the cell. Lateral cell wall may be deposited either at one or two growing zones or by diffuse intercalation of cell wall material on the cell surface. The results on *cocci* should be considered separately, since they belong to a different morphogenetic system than rod-shaped organisms. Cole and Hahn (1962) first demonstrated, with the immunofluorescence technique, that the addition of new cell surface in *S. pyogenes* occurred at a restricted number of zones per

cell. New wall appeared to be added at the coccal equator. In *S. faecalis* (Higgins et al., 1971) a single equatorial growth zone exists. Peptidoglycan is deposited in an outward direction, starting from the equatorial ring. The cross wall peels apart into two layers of peripheral wall. Shockman et al. (1974) proposed a model for cell wall growth in *S. faecalis* in which the wall synthetic activity at the leading edges of the nascent cross walls is responsible for both surface enlargement and cross wall formation. Probably all Gram-positive *cocci*, dividing in one plane, have one to three wall enlargement sites per nascent diplococcus (Shockman et al., 1974).

The consistency and agreement of results obtained with *streptococci* contrasts sharply with the notable lack of agreement between the results of attempts to localize wall enlargement sites in rod-shaped Gram-positive and in Gram-negative organisms. From observations on the growth of *E. coli* Donachi and Begg (1970) developed the concept of the "unit cell". A unit cell is the smallest cell of a given strain that can exist in any growth condition. This unit cell has a single membrane growth site, located at one pole. Formation of new membrane takes place at one side of this site only by asymmetrical growth. The number of sites increases in proportion to the size of the cells, so that in longer cells the growth becomes symmetrical. Mauck et al. (1971) suggested that cell wall growth occurs by uniform deposition of new wall over the whole surface. This suggestion was based on experiments in which cells of *B. megaterium* were labeled with radioactive diaminopimelic acid and the distribution of label among the daughter cells was observed autoradiographically. However, Ryter et al. (1973) found in *E. coli* a well defined growth zone on the bacterial surface. Radioactive cell wall precursor (diaminopimelic acid) was shown to be taken up at a well defined zone of the peptidoglycan sacculus. From this zone, rapid mixing of labeled peptidoglycan with preexisting peptidoglycan and distribution over the whole cell surface occurred. The changes in cell wall structure of a penicillinase-constitutive strain of *B. cereus*, exposed to penicillin followed by removal of penicillin with penicillinase, suggested that longitudinal extension occurs by addition of cell wall material to a

large and continuously increasing number of growing points, uniformly distributed over the cylindrical surface (Highton and Hobbs, 1972). By studying the cell wall morphology of *B. subtilis* and *B. megaterium* during recovery from amino acid starvation (which thickens the cell wall), Frehel et al. (1971) found indications that wall synthesis occurs at a large number of sites, uniformly distributed along the cylindrical part of the cells. The polar regions, however, were devoid of these sites.

In *B. subtilis* teichoic acids are linked covalently to the peptidoglycan. In absence of inorganic phosphate, teichoic acid synthesis can be turned off, whereas after addition of phosphate the teichoic acid synthesis is resumed. An examination of the teichoic acid-peptidoglycan complex, obtained from cells during a period of phosphate limitation followed by resumption of growth, has shown that the glycan chains adjacent to the chains linked covalently to teichoic acid, represent a random selection of new and old peptidoglycan chains. This is indicative for a random intercalation of new and old chains during cell wall growth (Mauck and Glaser, 1972). Experiments with a temperature-sensitive rod-mutant of *B. subtilis* which grows as a rod at 30° and as a sphere with thick cell wall at 45°, showed by direct visualization of the rod-morphology along the cell length during transition from 30° to 45°, that a single growth zone occurred (Mendelson and Reeve, 1973).

There is no agreement whether the wall enlargement in bacterial rod-shaped organisms occurs localized or at many growth sites. There is a preponderance of reports that favor a large number of growth sites on the cell wall or the cell membrane (Shockman et al., 1974).

1.2.9. Turnover of cell wall peptidoglycan

The question whether the bacterial cell wall is a metabolically stable structure or is subject to turnover is important in problems concerning cell wall synthesis. Turnover of cell wall peptidoglycan has been studied in a number of organisms by Boothby et al. (1973). *L. acidophilus* showed during logarithmic growth a turnover of 30% per

generation time. The turnover per generation time was not significantly decreased in slowly growing cultures. This makes a relation between growth rate and turnover improbable. It was only slightly decreased in a mutant in which autolytic activity was strongly depressed. This suggests that autolytic activity and turnover are not strongly interdependent. Inhibition of protein biosynthesis completely prevented peptidoglycan turnover and the cell wall was thickened. Mauck et al. (1971[b]) observed extensive turnover during logarithmic growth in the peptidoglycan of *B. subtilis*, where the rate of turnover was 50% per generation time. In *B. megaterium* during logarithmic growth a turnover rate of 30% per generation time was apparent (Mauck et al., 1971[b]). In *B. subtilis* the turnover was independent on the growth rate but freshly synthesized wall did not become available for turnover for 0.5-1 generation time (Mauck et al., 1971[b]). The products of turnover in *B. subtilis* were the products of cell wall cleavage by N-acetyl-muramyl-L-alanine amidase and could be isolated from the culture medium.

However, turnover does not seem to be a general phenomen for bacterial cell wall. Turnover of peptidoglycan was not observed in *E. coli* (Van Tubergen and Setlow, 1971), in a diaminopimelic acid-requiring mutant of *B. megaterium* during vegetative growth (Pitel and Gilvarg, 1970), and in *S. faecalis* (Boothby et al., 1973). A possible correlation between the turnover rate of peptidoglycan and the fraction of cell wall synthesis related with wall enlargement may exist, but there does not seem to be a correlation with cell wall thickening. After inhibition of protein synthesis in *L. acidophilus*, turnover of peptidoglycan ceased, but the cell wall thickened (Boothby et al., 1973).

There are probably no preferential sites on the bacterial surface for turnover. Fan et al. (1974) showed that in *B. subtilis* there is no preferential turnover in side walls over turnover in polar regions. The wall of this organism shows turnover over the entire surface of the cell.

1.2.10. Synthesis of cell wall peptidoglycan and cell division

After division of a bacterial cell each daughter cell should be the half of the parent and contain a copy of the parent genome. The coupling between cell surface growth (peptidoglycan synthesis), cell septum growth (also peptidoglycan synthesis) and DNA synthesis is not yet understood in molecular biological terms. However, these processes and DNA segregation must be co-ordinated. Shockman et al. (1974) proposed a scheme for the regulation of surface enlargement and cell division in *streptococci*. In rod-shaped organisms, the place where septum formation will start is determined by certain factors, because the septum is always in the middle between the two polar ends. Fiedler and Glaser (1973) proposed a diffusible inhibitor of septum synthesis which is subject to random degradation and is produced by the ends of the cell. The concentration of this inhibitor should be at a minimum in the middle of the cell to allow septum formation. The separation of the daughter cells after cell division is completed by formation of the septum in a process in which autolytic enzymes are probably involved. Bacterial mutants with low autolytic activity (Forsberg and Rogers, 1971; Fan and Beckman, 1972) showed surface growth but the cells did not separate well, resulting in formation of chains and clusters. Addition of autolytic enzymes to the medium did separate the cells and resulted in normal growth. These results suggest a possibly important role of autolytic enzymes in cleaving the peptidoglycan bonds which prevent the cell separation in the process of cell division.

1.2.11. Synthesis of cell wall peptidoglycan and cell shape

The synthesis of bacterial cell wall has two different aspects: the synthesis of septum and the synthesis of lateral cell wall. The cell shape is determined by the balance between cell elongation and the quantity of septum material deposited. There are no clear indications that the lateral cell wall and the septum are synthesized by different enzymes. Fan et al. (1972) demonstrated that the cylindrical zones of *B. subtilis* cells were structurally different from those at the

hemispherical caps. This could be an indication for involvement of different enzymes. However, Fan and Beckman (1973) were able to show that these structural differences are probably due to modifications after the synthesis rather than to involvement of different enzymes. An indication for involvement of different enzymes for formation of septum and lateral cell wall was given by Schwarz et al. (1969). They found that in *E. coli* the septum-synthesizing system showed preferential sensitivity to penicillin over the lateral wall synthesizing system.

The bacterial shape itself is fixed by the peptidoglycan network, also called sacculus. Upon isolation this sacculus retains exactly the size and shape of the organism (Weidel et al., 1960). Degradation of the cell wall peptidoglycan will affect the cell shape. It is an interesting question whether the cell shape of the organism or the sacculus is determined by its chemical composition or by morphogenetic systems. In *E. coli* significant variation in the composition of the peptidoglycan had no effect on the form of the sacculus and the shape of the cell (Schwarz and Leutgeb, 1971). This suggests that there is no simple correlation between chemical composition and shape of the cell. Spheroplasts (organisms of which the wall is removed) of rod-shaped organisms were allowed to form a new stable sacculus. This sacculus, however, had the spherical shape of the spheroplast in stead of a rod-shape. Despite the different shape of the sacculus the chemical composition did not appear to be different from that of a rod-shaped sacculus (Schwarz and Leutgeb, 1971). These results suggest the presence of a morphogenetic apparatus regulating the cell shape. Mutants of *B. subtilis* which grow as round forms in certain media appeared to have the same chemical composition, degree of cross-linking and length of peptidoglycan chains as the rod-shaped parent (Rogers et al., 1971). Cells of *Arthrobacter crystallopoietes* can undergo characteristic changes in the morphology during growth from spheres to rods. No large differences between the chemical composition of sphere and rod peptidoglycan occurred but the rod peptidoglycan had longer chains, and was more homogeneous in length than the sphere peptidoglycan (Krulwich et al., 1967). *Myxococcus xanthus* occurs, depending on the stage of life cycle as a rod-shaped vegetative

cell or as a spherical microcyst. Both cell types contained the same percentage of peptidoglycan and the overall chemical composition was similar. However, during transition from ovoid to sphere there was a transient decrease of cross-linking (White et al., 1968).

These experimental findings demonstrate that there is no clear relation between the chemical composition of the sacculus and the cell shape. In certain cases the changes in cell shape are paired with changes in macromolecular composition, but there is not always a strict coupling.

1.2.12. Inhibition of cell wall peptidoglycan biosynthesis

A number of antibiotics or growth inhibitory conditions affect the biosynthesis of peptidoglycan. Since in our experimental approach inhibition of cell wall biosynthesis is used as a tool we shall discuss the mechanism of action of the antibiotics and growth inhibitory conditions applied. Fig. 4 gives a schematic representation of the most important reactions in the biosynthesis of peptidoglycan and of the sites at which certain antibiotics and growth conditions will affect this biosynthesis.

a. Penicillin G

Penicillin G is an antibiotic able to kill growing, but not resting bacteria (Hobbey et al., 1942). The inhibition of growth can be accompanied by the accumulation of uridine nucleotides in the culture medium. These uridine nucleotides are known as precursors in the process of cell wall peptidoglycan biosynthesis (Park, 1952). Current understanding of the interaction of penicillin with the microbial cell indicates that it reacts with multiple components on the cell surface of which at least one or a number is involved with lethality. The binding of penicillin to bacterial membranes has been studied extensively (Duerkson, 1964; Rogers, 1967; Suginaka et al., 1972). It has been established that a small amount of penicillin is firmly bound by components of the bacterial membrane designed as "penicillin-binding" components (Blumberg and Strominger, 1972, 1974). Presumably one of

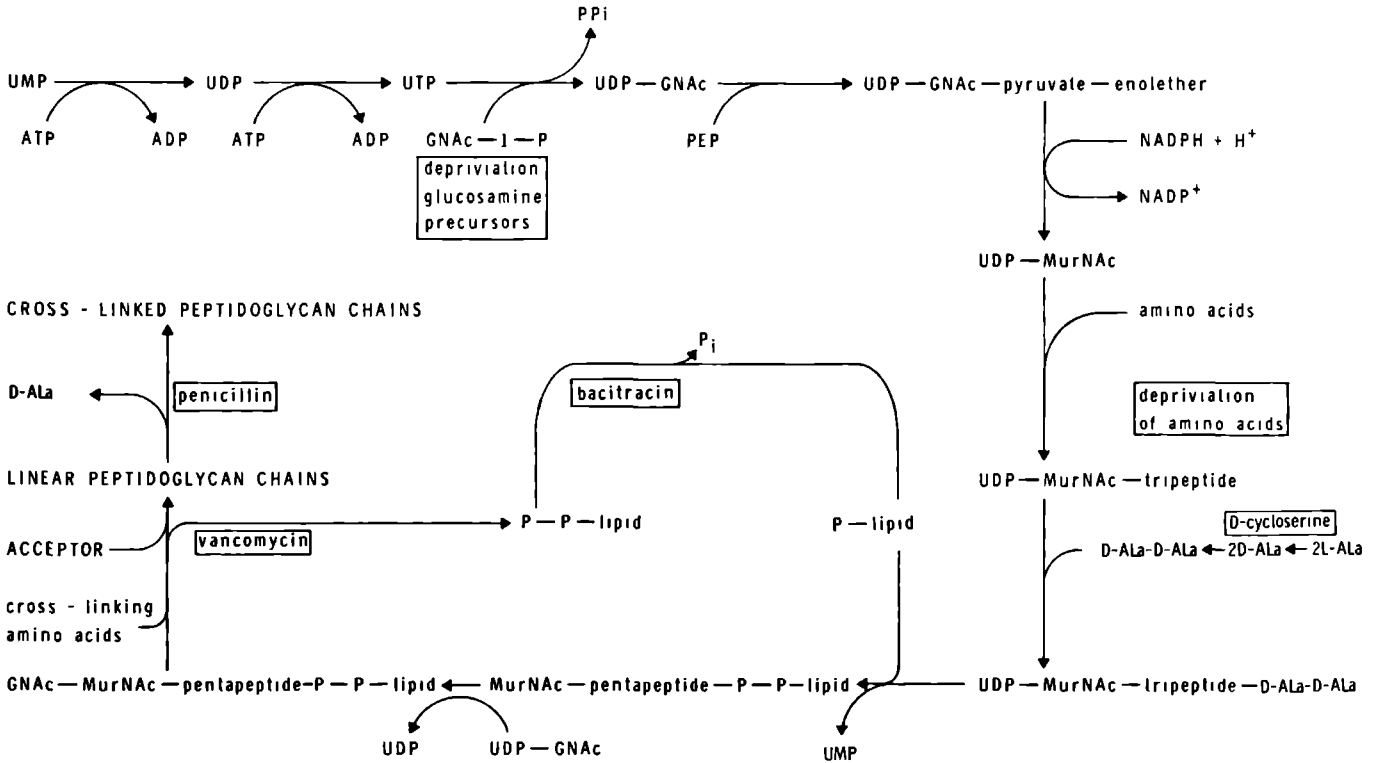

Fig. 4. Inhibition of peptidoglycan biosynthesis

these components is the transpeptidase which catalyzes cross-linking of the peptidoglycan. Strong evidence that penicillin acts on the level of transpeptidation emerges from several studies. Tipper and Strominger (1968) were able to demonstrate directly an accumulation of non-crosslinked peptidoglycan monomers containing two D-ala molecules per peptide subunit after inhibition of cells by penicillin. Inhibition of the transpeptidation reaction has also been shown in vitro (Tipper and Strominger, 1968; Izaki et al., 1968; Wickus and Strominger, 1972; Mirelman and Sharon, 1972). Total incorporation of UDP-MurNAc-pentapeptide into a polymer product is not affected by penicillin. However, the release of D-ala (a product of the cross-linking reaction) was fully inhibited. The product consisted of non-crosslinked monomer units with uncleaved D-ala-D-ala termini.

Studies on the inhibition of microbial growth by penicillin suggested that inactivation of penicillin-sensitive targets may result from the opening of the β-lactam ring of penicillin and penicillinoylation of certain enzymes (Izaki et al., 1966, 1968). It has been demonstrated indeed that penicillin is bound in particulate systems as penicillinoyl derivatives. However, it has not been proved that the penicillin binding sites of an organism are identical with the killing sites (Lawrence and Strominger, 1970).

Although experimental evidence on the mechanism of inhibition of the transpeptidation reaction is still incomplete, Strominger and Tipper (1965) proposed a plausible hypothesis. This hypothesis is based upon a structural analogy between the β-lactam ring of penicillin and the D-ala-D-ala terminus of the non-crosslinked peptidoglycan. Penicillin may be considered as an analogue of D-ala-D-ala from a structural standpoint. Since penicillin is a cyclic dipeptide of L-cysteine and D-valine, ring closure fixes the penicillin molecule in a single conformation. One of the conformations of D-ala-D-ala is nearly identical with that of penicillin. It was postulated that penicillin has almost exactly the conformation of the D-ala-D-ala terminus of a peptide subunit fixed to the binding site of the transpeptidase. Because of its fixed molecular conformation penicillin has a very high affinity for the substrate binding site. The transpeptidase normally

catalyzes a reaction in which the D-ala-D-ala bond is cleaved, presumably mediated by a substituted D-alanyl-enzyme complex. The peptide bridge of the peptidoglycan is closed by transfer of the D-alanyl-substituted residue to the free amino acid of the interpeptide bridge. In penicillin the highly reactive amide bond of the β-lactam ring is the equivalent of the peptide-bond of D-ala-D-ala. When fixed to the transpeptidase a facile acylation of the transfer site would occur with opening of the β-lactam ring. A penicillinoyl-enzyme is formed and the enzymatic process of transpeptidation is inactivated.

Besides the inhibition of transpeptidation, penicillin is known to inhibit another enzyme which plays a possible role in the biosynthesis of the cell wall: D-ala carboxypeptidase (Leyh et al., 1971, Umbreit and Strominger, 1973[a]). The D-ala carboxypeptidase cleaves the terminal D-ala residue from the pentapeptidase subunit in non-cross-linked peptidoglycan, in uridine nucleotide peptide and in several other substrates. The result of the cleavage is a tetrapeptide unit, incapable of serving as a transpeptidation donor. One possible role of the D-ala carboxypeptidase is perhaps the regulation of the cross-linking in the cell wall (Izaki et al., 1968). It has been shown that the D-ala carboxypeptidase activity differed appreciably from the penicillin killing site. Inhibition of the enzymatic activity by up to 95% had no measurable effect on the growth of the organism and the degree of cross-linking (Blumberg and Strominger, 1971; Sharpe et al., 1974).

b. Vancomycin

Vancomycin interferes with the biosynthesis of cell wall peptidoglycan in Gram-positive organisms (Reynolds, 1966). The molecular structure of vancomycin still remains to be delineated. It is, however, known that vancomycin consists of glucose, aspartic acid, N-methyl-leucine, phenols and chlorophenol molecules (Perkins and Nieto, 1974). Vancomycin is rapidly adsorbed from solutions by Gram-positive bacteria. The adsorbed material is not removed by simple washing procedures (Jordan, 1965). The largest fraction is adsorbed to the cell wall.

The stage at which vancomycin interferes with the peptidoglycan biosynthesis by cell-free membrane preparation has been studied by several authors (Anderson et al., 1965; Anderson et al., 1967[a]; Anderson et al., 1967[b]; Reynolds, 1971[a]; Reynolds, 1971[b]). Vancomycin inhibits the biosynthesis of peptidoglycan. It did not influence transfer of radioactivity from labeled UDP-acetylmuramyl pentapeptide precursor to lipid-intermediate. However, vancomycin was not effective in preventing the transpeptidation-dependent incorporation of radioactive glycine into a wall membrane preparation of *S. aureus* (Mirelman and Sharon, 1972). This suggests that vancomycin is active in the inhibition of peptidoglycan synthesis at a stage between transpeptidation and transfer of peptidoglycan from the lipid-intermediate to a cell wall precursor.

About the molecular site of action of vancomycin little is known. However, vancomycin is known to have a characteristic affinity for peptides. Trichloroacetic acid extracts of certain Gram-positive organisms inhibited with vancomycin, contained UDP-N-acetyl-muramyl-pentapeptide precursors which were complexed with vancomycin in nearly equimolar proportions (Chatterjee and Perkins, 1966). It was shown that the only part of the peptidoglycan precursor molecule that was essential for binding was the acyl-D-alanyl-D-alanyl terminus. The D-configuration was an essential factor (Perkins, 1969). Another requirement for vancomycin binding was the presence of a free carboxyl terminus in the peptide part of the precursor molecule. When this was not present the complexation with vancomycin was prevented. The complexation between the peptide and vancomycin could not be disturbed by the use of fairly high concentrations of disrupting agents as urea, sodium dodecylsulphate, KCl (Nieto and Perkins, 1971). indicating a firm interaction between the two components.

c. Bacitracin

Bacitracin is a polypeptide antibiotic which is known to inhibit synthesis of bacterial cell wall and to induce the accumulation of UDP-MurNAc-pentapeptides in certain micro-organisms. Bacitracin is active in inhibiting the dephosphorylation of the C_{55}-isoprenyl pyro-

phosphate.

$$C_{55}\text{-isoprenyl-P-P} \rightarrow C_{55}\text{-isoprenyl-P} + P_i$$

This reaction, which is catalyzed by the enzyme C_{55}-isoprenyl-pyrophosphatase, serves to regenerate the lipid carrier needed for transport of the peptidoglycan precursor through the membrane. In the presence of bacitracin this reaction is prevented and C_{55}-isoprenyl pyrophosphate accumulates. The peptidoglycan biosynthesis is blocked by lack of regenerated C_{55}-isoprenyl-P (Siewert and Strominger, 1967). The antimicrobial activity of bacitracin is stimulated by various metal ions. Metal ions may even be essential for antimicrobial activity (Cornell and Guiney, 1970). Proof for a direct formation of a complex between the bacitracin and the C_{55}-isoprenyl pyrophosphate was given by Stone and Strominger (1971). Various divalent cations can participate in complex formation, whereas monovalent cations are ineffective. The efficiency of divalent cations varied widely: Zn^{2+} is far more effective than Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+} or Cd^{2+}.

It was also shown that a correlation exists between the binding constant of various bacitracin peptides with the C_{55}-isoprenyl pyrophosphate and their antimicrobial activity (Storm and Strominger, 1973). These results suggested that bacitracin inhibits dephosphorylation of the C_{55}-isoprenyl pyrophosphate by formation of metal ion-mediated complexes with the isoprenyl pyrophosphate, and so preventing the pyrophosphatase action. In fact, a one-to-one complex of bacitracin with the C_{55}-isoprenyl pyrophosphate was found in the presence of a divalent metal ion. The pyrophosphate group of the C_{55}-isoprenyl pyrophosphate appears to fit into a pocket in the bacitracin molecule and the ion may be a ligand between the antibiotic and this polar end of the lipid.

The action of bacitracin is not merely restricted to the cell wall synthesizing mechanism. Bacitracin clearly also damages protoplasts and L-forms (which have no peptidoglycan) of certain organisms (Hancock and Fitz-James, 1964; Snoke and Cornell, 1965). Bacitracin appears to have also some effect on the membrane structure. Studies of Storm

and Strominger (1974) and Storm (1974) suggested that bacitracin interacts specifically with the C_{55}-isoprenyl pyrophosphate in the membrane without interacting strongly with other membrane lipids.

d. D-cycloserine

D-cycloserine inhibits the biosynthesis of cell wall peptidoglycan. The inhibition of peptidoglycan synthesis can be accompanied by the accumulation of the peptidoglycan intermediary UDP-MurNAc-tripeptide (Strominger et al., 1959). Two large targets for inhibition by D-cycloserine in the synthesis of peptidoglycan are known. D-cycloserine inhibits competitively alanine racemase as well as D-alanyl-D-alanine synthetase and inhibits in this way the production of an essential part of the peptidoglycan precursor: UDP-MurNAc-pentapeptide. As a result of studies on alanine racemase from *S. aureus*, Roze and Strominger (1966) proposed a hypothesis for the mechanism of cell wall peptidoglycan biosynthesis by D-cycloserine. D-alanine racemase is competitively inhibited by D-cycloserine because of structural analogy of D-cycloserine and D-alanine. D-cycloserine has a conformation which is identical with one of the many possible conformations of D-ala and L-ala. This conformation is preferred for binding of the substrate to the enzyme. Since D-cycloserine has a rather fixed conformation, due to its closed ring structure, it remains in this conformation, which is preferred for binding to the enzyme. Hence, it is up to 100 times as effectively bound compared with the natural substrate. L-cycloserine is no inhibitor since it does not have the preferred conformation for optimal substrate binding. However, there are indications that this hypothesis is not valid for all bacterial species (Lambert and Neuhaus, 1972).

e. Amino acid deprivation

Deprivation of certain amino acids may also lead to inhibition of cell wall peptidoglycan synthesis. This will only occur in those organisms in which deprivation of amino acids will lead to a shortage of amino acids needed for the synthesis of peptidoglycan precursors. However, in these organisms inhibition of cell wall biosynthesis will be coupled with inhibition of protein biosynthesis.

f. Deprivation of glucosamine precursors

N-acetyl-glucosamine-1-phosphate is an essential precursor for the synthesis of cell wall peptidoglycan. In certain auxotrophic organisms deprivation of glucosamine precursors may lead to inhibition of cell wall peptidoglycan synthesis. An example is *B. bifidum* var. *pennsylvanicus* which needs certain glucosamine-containing derivatives for growth.

Concluding we have shown the following inhibitory mechanisms, active at different sites in the cell and resulting in inhibition of the peptidoglycan synthesis:

(i)	Intracellularly	: deprivation of glucosamine precursors
		deprivation of amino acids
		D-cycloserine
(ii)	Cytoplasmic membrane	: bacitracin
		vancomycin
(iii)	Extracellularly	: penicillin

1.3. CHARACTERISTICS OF BIFIDOBACTERIUM BIFIDUM VAR. PENNSYLVANICUS

1.3.1. Isolation

Bifidobacterium bifidum var. *pennsylvanicus* is an organism belonging to the genus: *Bifidobacterium*. The bifidobacteria were first described by Tissier (1899). The organism occurs as Gram-positive, curved, often bifid rods and is predominant in stools of breast-fed infants. The bifidobacteria are non-motile, asporogenous and mostly anaerobic bacilli. In stool of bottle-fed infants the number of bifidobacteria is smaller compared to the stools of breast-fed infants (Poupard et al., 1973). Clinical observation, that breast-fed infants were apt to be less susceptible to infection than bottle-fed infants, and the speculation that bifidobacteria might play a role in the colon in the resistance to infection, has prompted much scientific interest in the bifidobacteria.

Norris et al. (1950) developed a culture medium which was satisfactory for primary isolation and laboratory maintenance of at least some

strains of bifidobacteria. This was an important step in the development of the research of bifidobacteria since many difficulties were experienced in culturing and maintaining the organisms. Because of the suspicion that an ingredient of human milk might be important, György et al. (1955) added defatted human milk to the Norris medium. They isolated a strain of bifidobacteria which required human milk. It was originally named: *Lactobacillus bifidus* var. *pennsylvanicus*. Its name was later changed into *Bifidobacterium bifidum* var. *pennsylvanicus* (Veerkamp, 1969[b]). The organism could also be isolated from stools of bottle-fed infants and from vaginal secretions of pregnant women (György, 1953).

1.3.2. Growth factors for B. bifidum var. pennsylvanicus

B. bifidum var. *pennsylvanicus* shows only scant growth in the Norris medium when human milk is omitted. Addition of human milk propagates growth. In the presence of human milk the organism grows as a rod-shaped bacterium, while in the absence of human milk the rod-shape is transformed to bizarre, branched forms (Glick et al., 1960). The essential factors for growth which were present in human milk but not in cow's milk were identified as N-acetyl-D-glucosamine-containing saccharides. The growth factors are used by the organism as substrates for cell wall synthesis (Glick et al., 1960; O'Brien et al., 1960). The natural growth factors for *B. bifidum* var. *pennsylvanicus* have been intensively studied (György and Rose, 1955; Zilliken et al., 1954, 1955[a], 1955[b], 1956; Rose and György, 1957).

Certain N-substituted synthetic derivatives of D-glucosamine e.g. N-benzoyl-D-glucosamine, also promoted growth of the organism (Rose et al., 1954; Lambert and Zilliken, 1965). The glucosamine moiety of the synthetic growth factor is incorporated in the cell wall muramic acid and in the cell wall peptidoglycan (Glick et al., 1960; Lambert et al., 1965). Some of the N-substituted derivatives of D-glucosamine exhibited an activity exceeding that of the N-acetyl-D-glucosamine. A possible explanation was given by Veerkamp (1969[a]). The N-substituted synthetic derivatives are less rapidly converted to glucosamine-6-phosphate than

N-acetyl-D-glucosamine. Deamination of the glucosamine-6-phosphate is also inhibited by the 6-phosphate of some of the synthetic derivatives. Therefore the glucosamine moiety of the synthetic derivatives is used more efficiently for cell wall peptidoglycan synthesis. Veerkamp (1969[b]) also established that N-acetyl-D-glucosamine is rapidly converted to fructose-6-phosphate, which is for the greater part degraded to acetate and lactate. The glucosamine-6-phosphate synthetase activity was of no importance compared to the enzymatic activities of the catabolic route. The growth promoting effects of the synthetic N-substituted derivatives of D-glucosamine are thus due to an inefficiency of the system for hexosamine synthesis.

1.3.3. Carbohydrate metabolism

Bifidobacteria are characteristic in their fermentation of glucose. In *B. bifidum* var. *pennsylvanicus* the route of glucose degradation has been established by Veerkamp (1969[b]). The organism does not ferment glucose along the glycolysis- and hexosemonophosphate pathways found in homo- and heterofermentative lactic acid bacteria.

1.3.4. Composition of the cytoplasmic membrane

The chemical composition of the cytoplasmic membrane of *B. bifidum* var. *pennsylvanicus* was established by Exterkate et al. (1970). The lipoprotein complex accounts for about 78% of the total membrane of which 70% is protein and about 8% is lipid material. The membrane lipids consist predominantly of glycolipids and phospholipids. The lipid composition of the organism was investigated by Exterkate and Veerkamp (1969) and Van Schaik and Veerkamp (1975). Eighteen phospholipids were detected, the major ones being: diphosphatidylglycerol, phosphatidylglycerol and a glycerophosphorylgalactosyldiglyceride. Mono-, di-, and tri-acyl-bis(glycerophosphoryl)glycerol, alanylphosphatidylglycerol and phosphatidic acid were present in smaller quantities. The glycolipids of the organism are all galactolipids. These galactolipids from about 50% of the total lipids. Besides mono-, di- and trigalactosyldiglycerides, a mono-acyl and a diacyl derivative of mono-

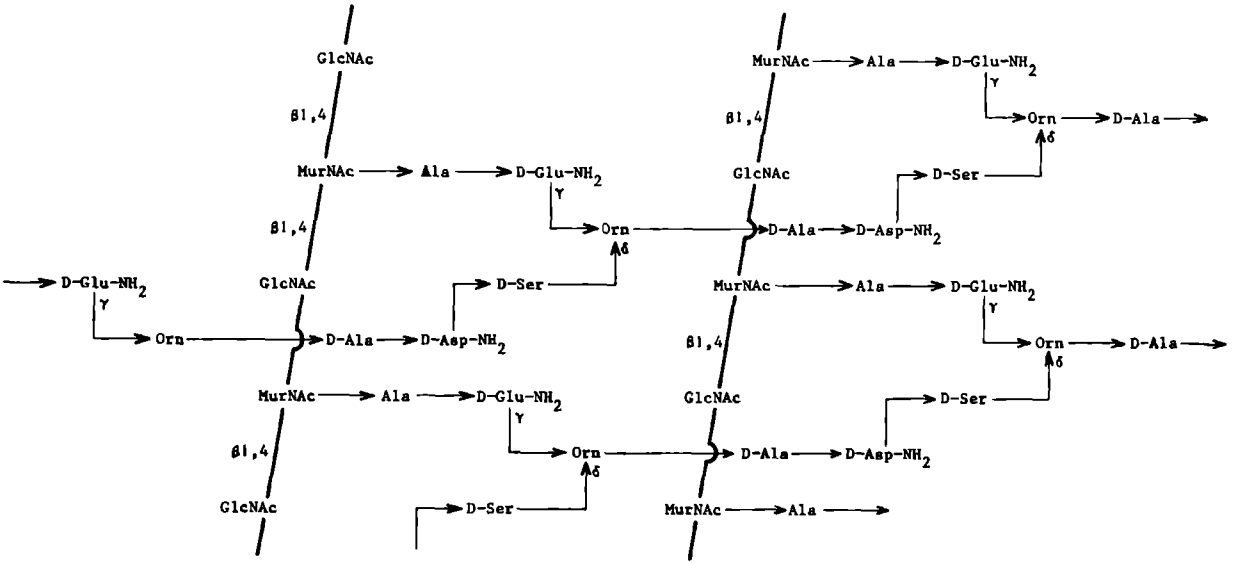

Fig. 5. Structure of the peptidoglycan of *B. bifidum* var. *pennsylvanicus* (Veerkamp, 1971)

galactosyldiglyceride, and a mono-acyl derivative of digalactosyldiglyceride were detected (Exterkate and Veerkamp, 1971). Monogalactosyl- and digalactosylmonoglycerides were present in small amounts. Mono-, di-, and tri-galactosyldiglycerides are only composed of galactopyranose units: diacylmonogalactosyldiglyceride and acyldigalactosyldiglyceride are galactofuranosides. The mono-acyl monogalactosyldiglyceride exists both as a galactofuranoside and galactopyranoside (Veerkamp, 1972). UDP-galactose is involved in the synthesis of these components (Veerkamp, 1974). The fatty acid composition of the lipids was also investigated by Veerkamp (1972). No large differences were found between the fatty acid patterns of total lipids, cytoplasmic membrane lipids and cytoplasmic lipids and glyco- and phospholipids. Major constituents were the normal, even-numbered, saturated and mono-enoic acids. The percentage of lactobacillic acid and branched fatty acids was low.

1.3.5. The cell wall of B. bifidum var. pennsylvanicus

The chemical analysis of the cell wall has been described by Veerkamp et al. (1965). The cell wall contains, in addition to peptidoglycan large amounts of rhamnose, glucose and minor amounts of galactose. Also small amounts of glycerol and phosphorus have been identified, which suggests the presence of glycerol teichoic acid. The structure of the cell wall peptidoglycan has been proposed by Veerkamp (1971). The peptidoglycan contains equimolar amounts of N-acetyl-D-muramic acid, N-acetyl-D-glucosamine, D-isoglutamine, D-(iso)asparagine, D-serine, L-ornithine, L-alanine and D-alanine. The structure of the peptidoglycan is shown in Fig. 5. Chains consisting of alternating N-acetyl-D-glucosamine and N-acetyl-D-muramic acid form the polysaccharide backbone. To the muramic acid are linked tetrapeptide units, composed of N^{α}-L-alanyl-γ-D-isoglutamyl-L-ornithyl-D-alanine. Dipeptide bridges between the δ-amino group of ornithine on one chain and the carboxyl group of C-terminal D-alanine on the other chain exist. The dipeptide bridges are seryl-asparagine units. About a quarter of the (iso)asparagine is N-terminal, indicating that about 75% of the possible cross-links are realized.

CHAPTER 2

INCORPORATION OF RADIOACTIVE PRECURSORS INTO CELL WALL AND CELL MEMBRANE

2.1. INTRODUCTION

In this chapter some aspects of the influence of growth inhibition on cell wall and cell membrane formation in *B. bifidum* var. *pennsylvanicus* are described. Suitable precursors for the major components of cell wall and cell membrane were selected and incorporation of labeled precursors under normal conditions and during growth inhibition was determined. Inhibition of synthesis of macromolecular compounds was applied as a means to disturb the processes of cell wall or cell membrane formation. The aim of these experiments has been described in the general introduction. The results of representative experiments are shown.

2.1.1. Inhibition of protein biosynthesis

Inhibition of protein biosynthesis prevents synthesis of one of the most important elements of the cell membrane. Though the biosynthesis of enzymes in the cell will be prevented too, this does not imply that the capacity of all the existing enzyme systems is abolished immediately. Under such conditions the formation of membrane protein is severely disturbed. Inhibition of protein biosynthesis was effected with chloramphenicol, tetracyclin and actinomycin-D, being antibiotics with different modes of action. Chloramphenicol acts specifically on the biosynthesis of protein and not on nucleic acid synthesis. The polymerization of amino acids is inhibited stereospecifically by action on the 50 S ribosome subunit. Chloramphenicol inhibits also the release of terminated protein from the ribosome. Protein biosynthesis is inhibited in a secundary mode by the unavailability of ribosomes for protein biosynthesis (Morel et al., 1972). Tetracyclin binds to the 30 S ribosome subunit and prevents attachment of the transfer ribonucleic acid (tRNA). Since the tRNA supplies the amino acids for protein synthesis, the production of protein will be arrested (Morel et al., 1972). Actinomycin-D disturbs protein syn-

thesis primarily by complexing with the bacterial desoxyribonucleic acid (DNA) and inhibiting the DNA-dependent ribonucleic acid synthesis (Goldberg, 1965).

2.1.2. Inhibition of peptidoglycan synthesis

Formation of cell wall was inhibited by inhibitors of peptidoglycan synthesis: penicillin G, bacitracin, D-cycloserine, vancomycin and staphylococcin 1580. Inhibition was also effected by omitting the growth factor N-benzoyl-D-glucosamine from the culture medium of the organism. Their mode of action has been described in Chapter 1. Penicillin G prevents cross-linking of newly formed peptidoglycan. Bacitracin inhibits the regeneration of the lipid carrier in the membrane. D-cycloserine inhibits competitively the synthesis of nucleotide precursors for cell wall peptidoglycan. Vancomycin prevents formation of linear peptidoglycan chains. Deprivation of growth factor inhibits peptidoglycan synthesis by lack of precursors for the peptidoglycan.
Staphylococcin 1580 is produced by *S. epidermidis* 1580 strains. In our experiments a preparation was used which was isolated from the culture medium by $(NH_4)_2SO_4$ precipitation and subsequent dialysis is distilled water, followed by lyophilization (Jetten, 1973). Its effect is bactericidal but not bacteriolytic. In sensitive organisms biosynthesis of macromolecular components is inhibited. Staphylococcin appears to act directly on the membrane, though the exact mechanism of action is not known (Jetten, 1973).

2.1.3. Omission of sodium acetate and Mg^{2+} ions from the culture medium

Growth inhibition with a much less specific action on cell wall and cell membrane formation is introduced by omitting sodium acetate and Mg^{2+} ions from the culture medium. Although the effects are not well defined they may give useful information about interactions between cell wall and cell membrane formation.

Mg^{2+} ions are important factors in many processes involving ATP. The

greater amount is needed for an adequate ribosome function (Stannier et al., 1963). Mg^{2+} is also involved in maintaining the structural integrity and functional ability of the bacterial cell envelope (Tempest, 1969).
In other experiments sodium acetate has been omitted from the medium, which leads to a decrease of the Na^{+} content from 319 to 15 meq/l with concomitant reduction of the osmotic pressure. The osmolality of the culture medium decreases from 822 to 223 mosm/kg (from 18.4 to 5 atm). This reduction in osmolality implies a changed osmotic environment for the bacterial cell. We do not know the internal osmotic pressure of the cell, but for Gram-positive organisms this is about 20-30 atm (Eisenberg and Corner, 1972).
Reduction of external osmotic pressure implies adaptation or protection of the cell since lysis of the cell becomes an actual danger. In such a situation it is interesting to follow processes of membrane and cell wall formation, especially in relation to each other.

2.2. MATERIALS AND METHODS

2.2.1. Organism

The strain *Bifidobacterium bifidum* var. *pennsylvanicus* was originally obtained from Dr. P. György, General Hospital, Philadelphia, U.S.A. under the name: *Lactobacillus bifidus* var. *pennsylvanicus*.

2.2.2. Growth conditions

The organism was cultivated in a medium according to Norris et al. (1950) at 37°, under a gas phase consisting of 90% N_2 and 10% CO_2. Tables III a-c give the composition of the culture medium. The pH of fresh culture medium was always adjusted to 6.8.

TABLE IIIa. COMPOSITION OF THE NORRIS MEDIUM

Component	Quantities per liter	
Adenine	17.4	mg
Alanine	200.0	mg
p-Aminobenzoic acid	10.0	mg
Ascorbic acid	2.0	g
Asparagine	100.0	mg
Biotin	0.05	mg
Calcium pantothenate	0.2	mg
Cystine	200.0	mg
Ferrous sulfate ($FeSO_4.7H_2O$)	10.0	mg
Folic acid	10.0	µg
Guanine	12.4	mg
Lactose	35.0	g
Magnesium sulfate ($MgSO_4.7H_2O$)	200.0	mg
Manganese sulfate ($MnSO_4.H_2O$)	6.7	mg
Nicotinic acid	0.6	mg
Casein hydrolysate * (enzymatic)	5.0	g
Potassium phosphate, dibasic (K_2HPO_4)	2.5	g
Pyridoxine hydrochloride	1.2	mg
Riboflavine	0.2	mg
Sodium acetate, anhydrous	25.0	g
Sodium chloride	10.0	mg
Sorbitan mono-oleate (Tween 80)	0.5	ml
Thiamine hydrochloride	0.2	mg
Tryptophan	200.0	mg
Uracil	10.0	mg
Xanthine	10.0	mg
Human milk (skimmed)	20.0	ml

* Obtained from ICN Nutritional Biochemicals

TABLE IIIb. CONTENT OF AMINO ACIDS FROM CASEIN HYDROLYZATE AFTER ACID HYDROLYSIS

Acid hydrolysis was performed with 6 N HCl for 16 h at 100°.

Component	Quantities per 1 (mg)
Aspartic acid	29.7
Threonine	11.5
Serine	13.0
Glutamic acid	36.2
Proline	8.1
Glycine	2.8
Alanine	8.3
Valine	16.3
Methionine	7.2
Isoleucine	13.1
Leucine	32.4
Tyrosine	10.0
Phenylalanine	13.9
Lysine	25.0
Histidine	5.4
Arginine	11.5

TABLE IIIc. FATTY ACID COMPOSITION OF THE CULTURE MEDIUM

Values are derived from Veerkamp (1970)

Component	Quantities per 1 (mg)
Oleic acid	58.5
Palmitoleic acid	8.2
Palmitic acid	2.8
Myristic acid	2.5
Pentadecanoic acid	1.5
Linoleic acid	0.7

2.2.3. Maintenance of the organism

For the maintenance of the strain the organism was subcultured every two days. Growth on medium lacking human milk was checked as an additional control for the occurrence of infections.

2.2.4. *Cultivation and harvesting*

Culture media were sterilized at 120° for 15 min. Sterilized culture media were preincubated at 37° and inoculated with 5 ml of a 24 h-culture per 1 medium. Cells were grown to exponential phase for 10 h at 37°, and to stationary phase for 16 h at 37°. During cultivation a gas phase of 90% N_2 and 10% CO_2 was maintained over the culture fluid. Cells were harvested from the culture by centrifugation at 10 000 g during 15 min at 4°.

2.2.5. *Chemicals*

All chemicals used were of the highest commercial purity available. Lysozyme (E.C 3.2.1.17) and desoxyribonuclease (E.C 3.1.4.5) were obtained from Boehringer, Mannheim, W.-Germany.
Chloramphenicol and Na-penicillin G were obtained from Mycofarm, Delft, The Netherlands; bacitracin from Farmachemie, Delft, The Netherlands; vancomycin-HCl from Eli Lilly & Co, Indianapolis, U.S.A.; actinomycin-D from Merck, Sharpe & Dohme International, U.S.A.; tetracyclin-HCl from R.I.T., Rijswijk, The Netherlands; and D-cycloserine from Sigma Chemical Company, St. Louis, U.S.A. Staphylococcin 1580 was kindly provided by Dr. G. Vogels, Department of Microbiology, Faculty of Sciences, University of Nijmegen, The Netherlands. The activity of each preparation was determined empirically and expressed in arbitrary units (A.U.) (Jetten, 1973).
N-benzoyl-D-glucosamine was prepared according to the method of Konstas et al. (1959).
Instagel scintillation fluid and omnifluor were purchased from Packard Instruments Company Inc., Brussels, Belgium and New England Nuclear, Dreiechenhain, West-Germany, respectively.

2.2.6. *Radioactive compounds*

[1-^{14}C]oleic acid, specific activity: 58.9 mCi/mmol; [2-^{3}H]glycine, specific activity: 2 Ci/mmol; [1-^{14}C]palmitic acid, specific activity: 57.9 mCi/mmol; and D-[^{14}C]glucosamine, specific activity: 3 mCi/mmol,

were obtained from the Radiochemical Centre, Amersham, England. N-benzoyl-D-[^{14}C]glucosamine was prepared from D-[^{14}C]glucosamine and benzoylchloride according to the method of Konstas et al. (1959). The obtained specific activity was 0.021 mCi/mmol.

2.2.7. Measurement of radioactivity

Samples were assayed for radioactivity in a Tri-Carb Liquid Scintillation Spectrometer model 3380 with an absolute activity analyzer model 544 from Packard Instrument Company. A solution of 4 g omnifluor/l toluene was used for scintillation fluid. For aqueous samples Instagel scintillation fluid was used. To 1 vol of sample always 2 vol Instagel were added.
Autoradiograms were made by covering chromatograms with Kodak No Screen X-ray film.

2.2.8. Determination of osmolarity and pH

Osmolarity of fluids was determined on a freezing-point osmometer (Advanced Instruments Inc., Needham Heights, Mass., U.S.A.) which was calibrated to give a read out in mosm per kg of solvent.
The pH of the fluids was determined with a Philips PW 9408 Digital pH-meter. The pH of cultures was measured in the supernatant after centrifugation.

2.2.9. Determination of Na^+ and Mg^{2+} content

The Na^+ concentration of fluids was determined on an Eppendorf flame photometer. Standard solutions of NaCl were used for calibration. Mg^{2+} concentrations were determined on an Unicam SP 1950 Atomic Absorption Spectrometer. Standard solutions of $MgSO_4$ were used for calibration.

2.2.10. Microscopic observation

Cells, protoplasts and lysates were studied with a Leitz-Wetzlar

Dialux Phase-contrast Microscope with an oil-immersion objective.

2.2.11. Sonication

Sonication was carried out with a Bransson Sonifier model B-12, equipped with a microtip. The maximal energy output was used (65W). During sonication, samples were cooled in ice.

2.2.12. Measurement of culture density

The density of cultures was measured as the absorbance at 550 nm of culture samples. The samples were appropriately diluted in 0.9% NaCl and measured in 13 mm round bottom glass cuvettes. An absorbance of 1.00 corresponded to a dry weight of 0.44 mg/ml culture. Measurements were carried out on a Bausch & Lomb Spectronic 20 spectrophotometer.

2.2.13. Incubation of cells with labeled precursors and antibiotics

Incubation vessels were designed for incubation and sampling of cultures without disturbing the anaerobic conditions (Fig. 6). During the experiments the vessels were incubated in a water-bath at 37°.

Cells from 10 h cultures were harvested and resuspended in culture medium without human milk to an absorbance of about 1.0. During the incubation experiments the human milk was substituted by N-benzoyl-D-glucosamine in a concentration of 25 µg/ml.

In all the experiments described in this thesis the concentrations of the antibiotics were as indicated in Table IV. The effective concentrations for growth inhibition were determined in preliminary experiments. Staphylococcin was always added in a concentration of 50 A.U. per ml.

Preincubation at 37° was performed for 1 h. Normal and inhibited culture were tested at the same initial absorbance. Radioactive precursors were added in the following amounts per ml of medium: [^{3}H]glycine, 15.0 nCi; N-benzoyl-[^{14}C]glucosamine, 50.0 nCi; [^{14}C]oleic acid, 4.9 nCi; [^{14}C]palmitic acid, 3.2 nCi.

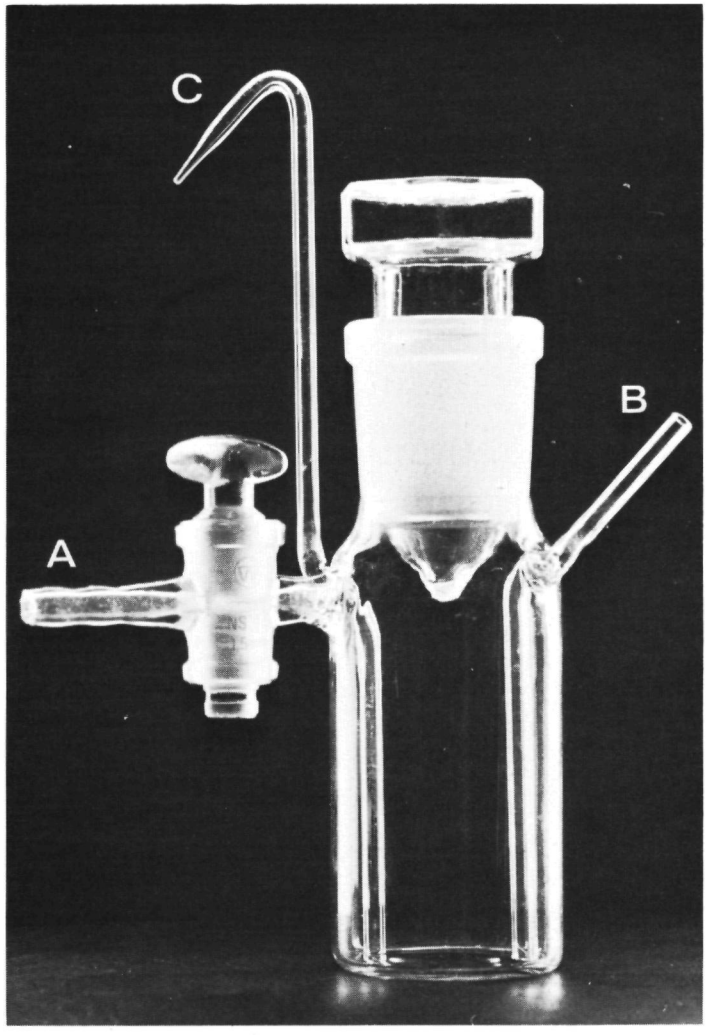

Fig. 6. Vessel for anaerobic incubation of cells. A: tap; B: gas inlet; C: sampling outlet.
N_2/CO_2 gas is introduced via B and led through the culture fluid inside the vessel while tap A is opened. After assessment of the anaerobic condition the gas flow is stopped and tap A is closed. Culture sample can be collected from C when gas is introduced into the vessel, while tap A is closed.

TABLE IV. CONCENTRATION OF ANTIBIOTICS

Inhibitor	Concentration (µg/ml culture medium)
Chloramphenicol	60
Tetracyclin	50
Actinomycin-D	10
Penicillin G	10
Vancomycin	20
D-cycloserine	80
Bacitracin	160

2.2.14. Determination of [^3H]glycine and [^{14}C]glucosamine incorporation

Samples of 2.5 ml culture were mixed with 2.5 ml 10% TCA of 0°. After 1 h incubation at 0° the precipitate was centrifuged in the cold and resuspended in 5 ml 5% TCA. Heat extraction during 30 min at 95° was carried out and the TCA-insoluble residue was collected by centrifugation. The residue was suspended in 5 ml 0.01 M NaOH, mixed with 10 ml Instagel, and radioactivity was determined by liquid scintillation counting, using a double label assay procedure.

2.2.15. Determination of [^{14}C]oleic acid and [^{14}C]palmitic acid incorporation

Samples of 2.5 ml culture were mixed with 2.5 ml culture medium without human milk of 0°. The cells were centrifuged 15 min at 10 000 g at 4° and washed two times with culture medium of 0°.
For the determination of [^{14}C]palmitic acid incorporation cells were washed after centrifugation once with cold culture medium and once with 1% Tween 40 in water of 0°. Washed cells were suspended in 5 ml 0.01 M NaOH. After mixing with 10 ml Instagel, radioactivity was determined by liquid scintillation counting.

2.2.16. Determination of incorporation in the cytoplasmic membrane fraction

Samples of 2.5 ml culture were added to 5 ml of culture medium without human milk of 0°. The cells were centrifuged 15 min at 10 000 g at 4° and resuspended in 5 ml 0.1 M phosphate buffer (pH 6.8), which contained 0.01 M Mg^{2+}.
Lysozyme was added in a concentration of 1.1 mg per ml buffer. Protoplast formation was effected by incubation at 37° during 40 min and controlled by microscopic observation of small samples. Protoplasts were desintegrated by sonication during 4 times 15 sec in ice. Desoxyribonuclease was added in a concentration of 12 µg per ml lysate and incubation during 5 min at 37° was carried out. Completeness of lysis was controlled by microscopic observation. Membranes were washed two times with 5 ml phosphate buffer-Mg^{2+} (pH 6.8) and taken up in 3 ml 1% SDS in water. For liquid scintillation counting 2 ml of the membrane suspension was adjusted to 5 ml with water and mixed with 10 ml Instagel. One ml of the suspension was used for protein determination (Lowry et al., 1951). Radioactivity was expressed as dpm per mg membrane protein.

2.2.17. Preparation of hot TCA-insoluble fraction after lysozyme treatment of cells

Samples of cultures (2.5 ml) were mixed with 2.5 ml of culture medium without human milk at 0° and centrifuged for 20 min at 10 000 g at 4°. Cells were resuspended in 2.5 ml 0.1 M phosphate buffer (pH 6.8) containing 0.01 M Mg^{2+}, and lysozyme was added in a concentration of 1.1 mg per ml buffer. Incubation at 37° during 40 min was performed. One vol 10% TCA (0°) was added and the mixture was incubated at 0° for 1 h. Heat extraction of the TCA-insoluble fraction and liquid scintillation counting were carried out as described in *2.2.14.*

2.2.18. Acid hydrolysis and chromatography of hot TCA-insoluble fraction

Hot TCA-insoluble fraction was prepared from [^{3}H]glycine-labeled cells

as described (*2.2.14*). The hot TCA-insoluble fraction was taken up in 6 N HCl and hydrolyzed 16 h at 100°. After filtration the hydrolyzate was evaporated *in vacuo* and dried *in vacuo* over NaOH pellets. The residue was dissolved in water and applied on thin-layer cellulose plates. The plates were developed with n.butanol-acetone-water (4:1:5, by vol) and n.butanol-pyridin-water (1:1:1, by vol) respectively. [^{3}H]glycine and unlabeled glycine were taken as referents. Radioactivity was detected by autoradiography and unlabeled glycine was detected with the ninhydrin reagent.

2.2.19. Extraction of lipids from cells after incorporation of [^{14}C]fatty acids

Samples of the culture were mixed with 1 vol of cold culture medium without human milk and centrifuged at 10 000 g during 15 min at 4°. Cells were extracted with chloroform-methanol (2:1, by vol). The lipid extract and the cell residue were separated by centrifugation and the extract was collected. For determination of radioactivity a sample was dried in a scintillation vial by an air system and 10 ml omnifluor-toluene scintillation fluid (4 g/l toluene) was added. For chromatographic procedures the extracts were concentrated by evaporation *in vacuo*.

2.2.20. Silica gel thin-layer chromatography

^{14}C-labeled lipids were analyzed by chromatography on silica gel G plates (0.25 mm thick). The plates were activated at 110° before use. Lipids were applied in small streaks and the plates were developed in benzene-diethylether-ethylacetate-acetic acid (80:10:10:0.2, by vol). Labeled and unlabeled fatty acids were taken as referents. After autoradiography ^{14}C-activity was quantitatively determined by liquid scintillation counting. Radioactive silica spots were suspended in 5 ml water and 10 ml Instagel.

2.3.1. Selection of a cell wall precursor

In the search for a suitable cell wall precursor, a precursor for the peptidoglycan was chosen. The cell wall peptidoglycan consists of two types of components: amino acids and carbohydrates. Since amino acids are components of the cellular protein, incorporation of amino acids into the cell may be not specific for peptidoglycan. A better alternative is formed by precursors of the carbohydrate moiety of the peptidoglycan. Lambert et al. (1965) demonstrated that a relative high amount of radioactivity of a number of growth factors containing ^{14}C-labeled glucosamine was incorporated into the muramic acid and glucosamine of the cell wall. One of the best growth factors is N-benzoyl-D-glucosamine. Maximal growth is reached with a concentration of 25 µg/ml N-benzoyl-D-glucosamine in the culture medium in absence of human milk (Lambert et al., 1965). For our studies N-benzoyl-D-[^{14}C]glucosamine was synthesized chemically and applied in incorporation studies. The incorporation of N-benzoyl-[^{14}C]glucosamine in a peptidoglycan-containing cell fraction was determined. Peptidoglycan is found in the insoluble fraction after extraction of the cells with hot 5% trichloroacetic acid (TCA) solution (Hancock and Park, 1958). When N-benzoyl-[^{14}C]glucosamine is used the [^{14}C]glucosamine incorporated into the peptidoglycan, is insoluble in hot TCA. Peptidoglycan formation was therefore determined by measuring the incorporation of radioactivity into the hot TCA-insoluble fraction of the cell. N-benzoyl-[^{14}C]glucosamine itself is soluble in hot TCA.

Theoretically the ^{14}C-activity from [^{14}C]glucosamine might be incorporated into products different from peptidoglycan. These products may comprise: teichoic acids, glycoproteins, fatty acids or polysaccharides. Teichoic acids are solubilized in hot TCA and do not contribute to radioactivity in the insoluble fraction (Armstrong and Baddiley, 1958). The amount of [^{14}C]glucosamine, incorporated in components different from peptidoglycan, may be estimated by a

lysozyme digestion experiment. Lysozyme specifically cleaves the glycoside bonds in the peptidoglycan, which is subsequently solubilized. Non-peptidoglycan components are not affected.

We labeled cells with [^{14}C]glucosamine and determined the hot TCA-insoluble fraction of the cells before and after lysozyme digestion. The fraction, not solubilized by lysozyme, contained 1.4% of the radioactivity measured in the entire hot TCA-insoluble fraction. This suggested that the label, incorporated into the hot TCA-insoluble fraction, was predominantly in peptidoglycan.
The amount of radioactivity incorporated into fatty acyl chains was estimated by the method of Cronan (1967). About 0.9% of the radioactivity incorporated into the hot TCA-insoluble fraction was found in the fatty acyl chains. On the basis of these results N-benzoyl-[^{14}C]glucosamine appeared to be a suitable cell wall precursor for our studies.

2.3.2. Search for a membrane protein precursor

A specific precursor for membrane protein does not exist. We had to search for a suitable protein precursor and find a technical means to discriminate between incorporation into the total cellular protein and into the protein of the cytoplasmic membrane.
The selection of a precursor for protein is described in this section whereas we shall describe in *2.3.4* a technique which discriminates between membrane protein and total cellular protein.

A potential precursor for the cellular protein in our study had to satisfy the following conditions:

(i) incorporation into the cellular protein should be quantitatively sufficient to allow accurate determination.

(ii) incorporation into the protein should be without structural modification of the precursor.

(iii) no incorporation should take place into components different from protein (e.g. peptidoglycan, lipids).

To find out which amino acid satisfied the first condition a number of amino acids was tested on their ability to be incorporated into the TCA-insoluble fraction of cells under normal growth conditions (Table V).

TABLE V. INCORPORATION OF [^{14}C]AMINO ACIDS

To 5 ml culture medium 0.1 µCi [^{14}C]amino acid was added. Cells were cultivated for 16 h and incorporation into TCA-precipitable material was determined.

Amino acid	Specific activity in culture medium (mCi/mol) ⋆)	Incorporation (dpm)
Aspartic acid	9.2	903
Lysine	11.7	892
Isoleucine	20.2	19860
Glycine	50.0	74855
Arginine	31.7	2321
Alanine	5.3	1361
Proline	28.9	1120
Glutamic acid	50.0	2960

⋆) The specific activity was calculated on basis of the total content of the amino acid in the culture medium.

The incorporation data are determined by

(i) the specific activity in the culture medium of the labeled precursors, which is determined by the composition of the casein hydrolyzate and the added amino acids.

(ii) the ability of the cells to take up and to incorporate the amino acids.

The combination of these factors apparently resulted in a relatively high incorporation of isoleucine and glycine. Glycine was chosen in

first instance for the study of protein synthesis since it was incorporated with a higher activity.
The second condition for a suitable precursor was that incorporation into the cell had to be without modification in structure. For this purpose, cells were labeled with [^{14}C]glycine and the TCA-insoluble fraction was hydrolyzed in 6 N HCl (*2.2.18*). Chromatography of the hydrolyzate in two different systems and subsequent autoradiography showed that nearly all radioactivity coincided with glycine.
There is no significant incorporation of glycine into fatty acids as determined by the method of Cronan (1967). Only 0.6% of the [^{14}C]glycine added was found in the fatty acid fraction. Glycine is not a component of the peptidoglycan in *B. bifidum* var. *pennsylvanicus* (Veerkamp, 1971). The results indicated that glycine was a suitable precursor for the cellular protein.

In our study we determined the incorporation of N-benzoyl-[^{14}C]glucosamine in peptidoglycan and [^{3}H]glycine in protein in a single assay. The same TCA precipitation procedure was applied since the protein fraction is also insoluble in hot TCA. To discriminate radioactivity [^{14}C]glucosamine and [^{3}H]glycine were used in our study.

2.3.3. Search for a membrane lipid precursor

Lipids are predominantly localized in the cytoplasmic membrane of Gram-positive organisms. A precursor which is incorporated into the cellular lipids, is in general also incorporated into the membrane lipids. Still there may be a certain amount of extra-membranous lipid present. We selected a lipid precursor for the cellular lipids and tried to discriminate between the membrane lipids and the total cellular lipids. In *B. bifidum* var. *pennsylvanicus* neutral lipids, glycolipids and phospholipids are present. Neutral lipids consist predominantly of : tri-, di-, and monoglycerides and free fatty acids. Glycolipids are predominantly galactosyldiglycerides (Veerkamp, 1972). Phospholipids may also contain carbohydrate moieties (Veerkamp and Van Schaik, 1974).

Despite the great variation in structure, some common structural elements exist between the different types of lipids. All lipids contain a glycerol moiety and fatty acyl chains.

In first instance glycerol was chosen as a lipid precursor. [^{14}C]glycerol was offered to the organism in the culture medium. However, in various experiments under different experimental conditions glycerol, even at a high specific activity, was not incorporated into the cellular lipids when added to growing cells. Possibly this was due to a deficiency in the organism of glycerokinase. This was confirmed by the finding that in cell-free, freeze-dried extracts of the organism no glycerokinase could be detected. We tried to label the lipids with [^{14}C]glycerol by offering the organism [^{14}C]glycerophosphate in the culture medium. Labeled glycerophosphate was synthesized from [^{14}C]glycerol with glycerokinase and added to the culture medium. However, the glycerolphosphate was also not incorporated into the cellular lipids. It appeared that this component was in fact not even taken up in the cell.

An alternative is provided by choosing fatty acids as precursors for lipids. In *B. bifidum* var. *pennsylvanicus* various fatty acids occur, the major ones being: oleic acid, palmitic acid and stearic acid (Veerkamp, 1970). There are no large differences in the fatty acid patterns of total membrane and cytoplasmic lipids, and glyco- and phospholipids. The major fatty acid in the lipids of organism, oleic acid was chosen as a lipid precursor. In normal cells, cultivated for 10 h in Norris medium, the oleic acid constitutes about 40% of the total fatty acids in all lipids. The organism can obtain oleic acid from the Tween 80 in the culture medium.

[^{14}C]oleic acid was incorporated into the cellular lipids, when added to growing cells. Cells, which were labeled with [^{14}C]oleic acid during logarithmic growth lost 99% of the radioactivity upon extraction with chloroform-methanol (2:1, by vol). This is an indication that all radioactivity incorporated was lipid-soluble. The incorporation was quantitatively sufficient to allow accurate determination.

Theoretically one might expect in general structural modifications of the [^{14}C]oleic acid in the cell. However, elongation of the chain or an increased unsaturation can not take place since the fatty acid fraction does not contain significant amounts of fatty acids with more than 18 C-atoms and more than one double bond. Chain shortening is also not likely, since the organism has no β-oxydation system.

2.3.4. Isolation of the cytoplasmic membrane fraction

To discriminate components in the membrane from components in the total cell, the membrane fraction had to be isolated. A general method for membrane isolation of Gram-positive bacteria can be summarized in the next stages.

(i) digesting the cell wall by lysozyme, resulting in protoplast formation
(ii) osmotic and sonic destruction of the protoplasts
(iii) digestion of DNA with DNase
(iiii) high speed centrifugation
(iiiii) repeated washing of the membrane sediment.

The complete procedure used for our experiments is described in *2.2.16.*

To obtain quantitatively reproducible results the isolation of the membrane had to satisfy the following conditions: (i) the method had to be standarized in all stages; (ii) the amount of membrane material had to be quantitated.
Condition (ii) appeared necessary because despite well standarized isolation techniques the amount of membrane material obtained in various identical experiments showed some variation. It was therefore decided to quantitate the amount of membrane by measuring the protein content. Incorporation of isotopes into the membrane fraction was then expressed per mg membrane protein according to Kahane and Razin (1968). This procedure yielded well reproducible results.

2.3.5. Control cells

Incorporation data from different identical experiments differ somewhat from each other, despite completely standarized procedures. This is probably due to the different phases of growth of the cells in different experiments. In our experiments, cells from 10 h cultures were used. Cultures were always inoculated with a fresh 24 h culture (5 ml to 100 ml medium). However, after inoculation cells are subjected to a lag phase of 3-4 h. Variations in the lag phase have consequences for the actual phase of growth after 10 h. The variation in lag phase is due to a complex of microfactors and is difficult to prevent. This problem is overcome if during all experiments, also experiments with control cells are carried out and if one relates the data obtained, to those for control cells from the same original culture.

2.3.6. Incorporation of precursors under normal conditions

In this section the results of an incubation experiment, in which no inhibitory conditions were applied, are described. Cells from a 10 h culture were harvested and resuspended in fresh culture medium of pH 6.8. After preincubation at 37°, labeled precursors were added. Detailed descriptions of the incubation procedures and radiochemical assays are found in section 2.1. In Fig. 7a the absorbance during the incubation period is shown. The absorbance plot allows a rough estimate of the bacterial growth. The absorbance almost doubles during 90 min incubation, indicating a doubling of the number of bacteria. However, it is important to note that the absorbance can also be influenced by the bacterial shape, so changes in absorbancy do not necessarily exactly reflect changes in the bacterial number (Günther, 1966).

The incorporation of N-benzoyl-[^{14}C]glucosamine into the peptidoglycan is shown in Fig. 7b. A linear increase of radioactivity incorporated is apparent from 0 to 40 min but after 40 min the incorporation rate decreased. This pattern for incorporation of N-benzoyl-glucosamine was found in many experiments. At the end of the incubation period the incorporation rate decreased, while the absorbance still increased.

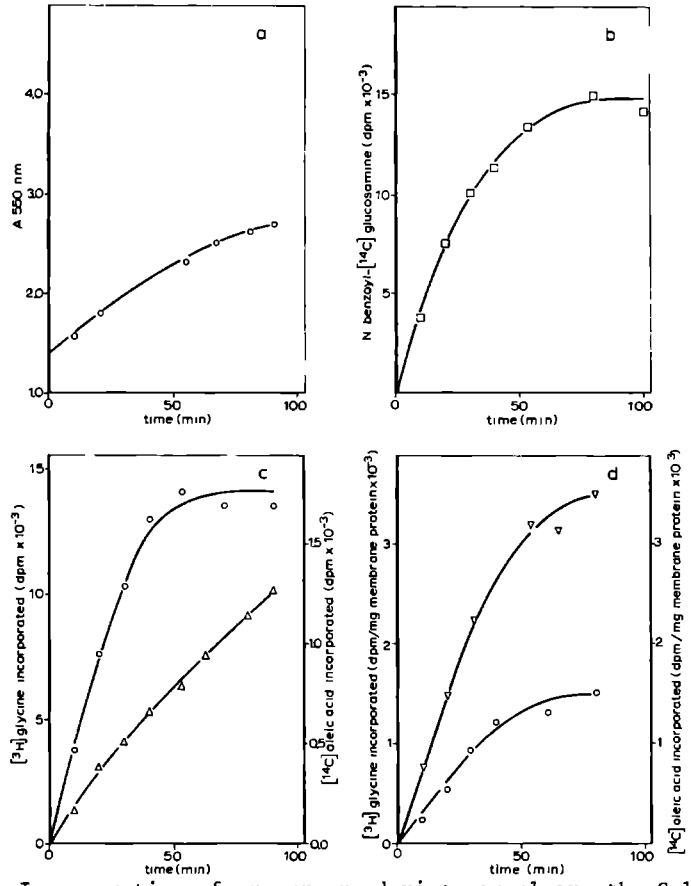

Fig. 7. Incorporation of precursors during normal growth. Cells were incubated with [^{3}H]glycine + N-benzoyl-[^{14}C]glucosamine and [^{14}C]oleic acid respectively. Incubation conditions, measurement of incorporation and determination of the absorbance are described in MATERIALS AND METHODS.

a. Absorbance of the culture;
b. Incorporation of N-benzoyl-[^{14}C]glucosamine into the hot TCA-insoluble fraction (-□-);
c. Incorporation of [^{3}H]glycine into the hot TCA-insoluble fraction (-Δ-); incorporation of [^{14}C]oleic acid into the cells (-o-);
d. Incorporation of [^{3}H]glycine into the membrane fraction per mg membrane protein (-Δ-); incorporation of [^{14}C]oleic acid into the membrane fraction per mg membrane protein (-o-).

Incorporation of [^{3}H]glycine is presented in Fig. 7c. An increase of radioactivity incorporated is found. [^{14}C]oleic acid incorporation into the entire cell (Fig. 7c) increased from 0 to 40 min but after this period the incorporation rate also decreased. This pattern for oleic acid incorporation was often found in our experiments.

Increase in incorporation of glycine and oleic acid into the membrane fraction is linear from 0 to about 40 min (Fig. 7d). A decrease of incorporation is apparent during the rest of the incubation period. The incorporation per mg membrane protein will undergo a rapid increase in the early incubation time under normal conditions. This value will give an impression of rates of synthesis and turnover in the membrane at a certain moment. After relatively long incubation times it will tend to become constant as the increasing incorporation equilibrates with the increase in the membrane protein mass. This is in fact what we observed in many experiments. After an increase in the incorporation of [^{3}H]glycine and [^{14}C]oleic acid the incorporation plots tended to decline.

2.3.7. Interpretation of the incorporation data

Concerning the incorporation data for the different precursors the following points are important for an interpretation of the effects during growth inhibition.

(i) the incorporation data should only be compared with data obtained for control cells from the same original culture;

(ii) the incorporation data give information about momentary changes in synthetic rates of components of cell wall and cell membrane, compared with normal growing cells;

(iii) the incorporation data do not necessarily reflect rates of net synthesis;

(iiii) the data for membrane components are expressed on the basis of the membrane protein content of the membrane fraction;

(iiiii) since growth inhibition may cause increased damage in the cell after long incubation times, only data for relatively short labeling periods should be considered.

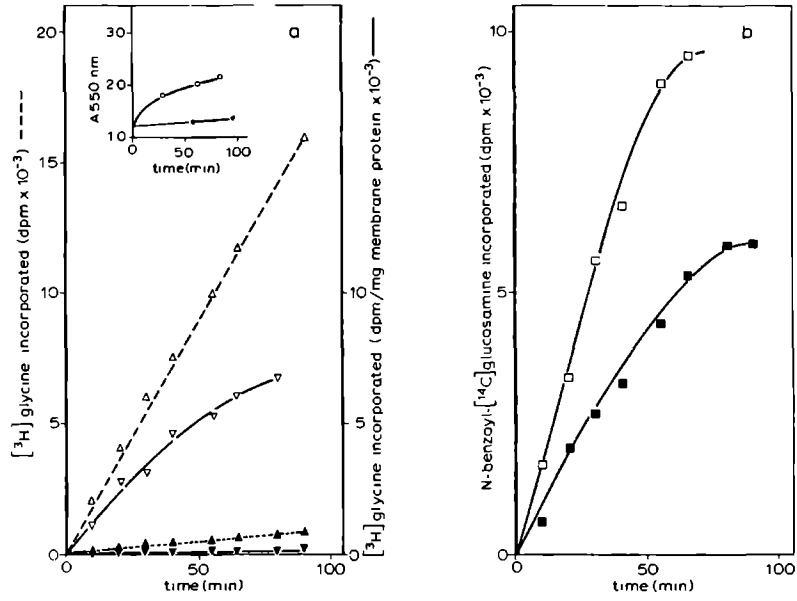

Fig. 8. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with chloramphenicol. Cells were incubated with radioactive precursors in the presence of 60 µg/ml chloramphenicol (closed symbols) and in the absence of chloramphenicol (open symbols). Incubation conditions, measurement of incorporation and determination of absorbance are described in MATERIALS AND METHODS (2.2).

a. Insertion: absorbance of the cultures;
   Incorporation of [^{3}H]glycine into hot TCA-insoluble fraction (Δ-Δ);
   incorporation of [^{3}H]glycine into the membrane fraction per mg membrane protein (∇-∇);

b. Incorporation of N-benzoyl-[^{14}C]glucosamine into hot TCA-insoluble fraction.

2.3.8. *Synthesis of peptidoglycan during inhibition of protein biosynthesis*

Chloramphenicol inhibited growth effectively (Fig. 8a) since the inhibited culture showed only a small increase in absorbance whereas the control culture showed an increase of nearly 100% after 90 min incubation. Protein synthesis was evidently inhibited. The incorporation rate of [^{3}H]glycine into inhibited cells was only 5% of that in control cells. Protein synthesis in the membrane fraction was also severely inhibited since no significant incorporation of [^{3}H]glycine was apparent. However, peptidoglycan synthesis continued

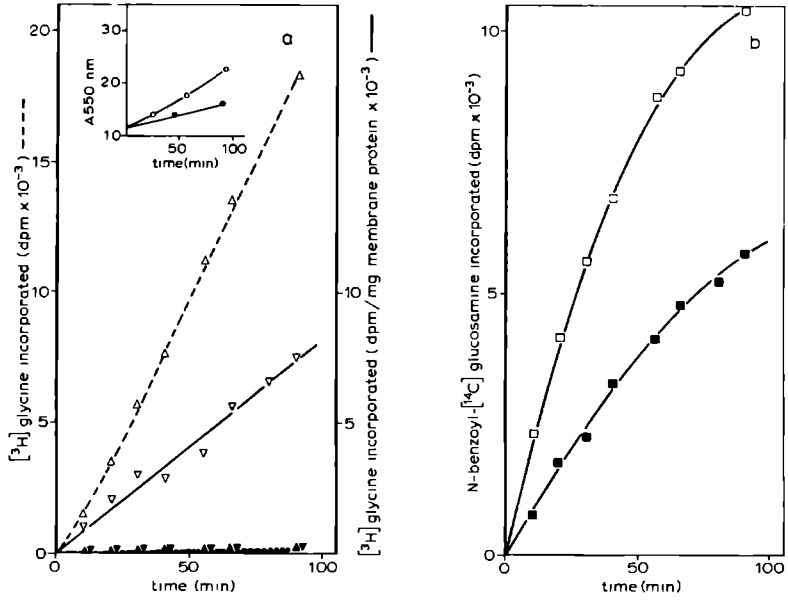

Fig. 9. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with tetracyclin. Cells were incubated with radioactive precursors in the presence of 50 µg/ml tetracyclin (closed symbols) and in the absence of tetracyclin (open symbols). Details are given in Fig. 8.

under these conditions (Fig. 8b). Incorporation of the precursor proceeded linearly from 0 to 60 min in both inhibited cells and control cells. The rate of incorporation of N-benzoyl-glucosamine in inhibited cells was about 50% of that in control cells.

The absorbance of the culture inhibited by tetracyclin rose only 25% compared with the control culture after 90 min incubation (Fig. 9a). Protein synthesis was effectively inhibited. Almost no incorporation of [^{3}H]glycine was found in the total cellular protein or in the membrane fraction, whereas a significant incorporation occurred in the control cells.

Incorporation of N-benzoyl-[^{14}C]glucosamine still proceeded under these conditions. In inhibited cells and in control cells a linear incorporation of N-benzoyl-glucosamine proceeded from 0 to 30 min. After 30 min the incorporation rates showed a small decrease. In the linear parts of the curve the rate of incorporation into inhibited cells was 51% of the rate in control cells (Fig. 9b).

The absorbance of the culture inhibited by actinomycin-D showed small increase during incubation whereas the control culture rose about 100% in absorbance (Fig. 10a). Protein synthesis was strongly inhibited. Synthesis of peptidoglycan continued under these conditions (Fig. 10b). Incorporation of N-benzoyl-[^{14}C]glucosamine proceeded linearly from 0 to 30 min in both cultures and showed a clear decrease of the incorporation rates after 30 min. Between 0 and 30 min the rate of incorporation into the inhibited cells was about 24% of the rate in control cells.

From these experiments it appeared that peptidoglycan synthesis continues despite nearly complete inhibition of the synthesis of cell protein and membrane protein. However, the rate of incorporation of N-benzoyl-[^{14}C]glucosamine was significantly lower in cells inhibited with actinomycin-D than in cells inhibited with chloramphenicol and tetracyclin (24% of the rate in control cells, compared with 50% for chloramphenicol and 51% for tetracyclin). It may be possible that a secondary effect of actinomycin action is on peptidoglycan synthesis. Observation of cells by phase-contrast microscopy, after incubation with actinomycin-D, showed a great number of twin cells.

This could mean that these cells were not able to complete cell division because of incomplete cell wall synthesis, which would be in agreement with the information obtained from the incorporation

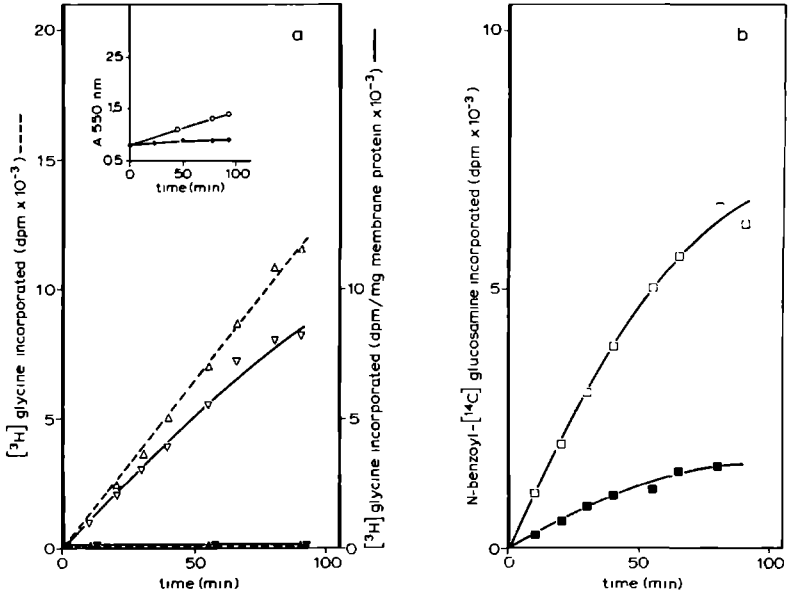

Fig. 10. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with actinomycin-D. Cells were incubated with radioactive precursors in the presence of 10 µg/ml actinomycin-D (closed symbols) and in the absence of actinomycin-D (open symbols). Details are given in Fig. 8.

During inhibition of protein biosynthesis considerable incorporation of N-benzoyl-glucosamine was found. Theoretically it might be possible, though unlikely, that the N-benzoyl-[^{14}C]glucosamine was also incorporated into non-peptidoglycan products. Hence, the fraction of non-lysozyme-digestible products in N-benzoyl-[^{14}C]glucosamine labeled material was determined (*2.2.17*). The results are presented in Table VI.

TABLE VI. INCORPORATION OF N-BENZOYL-[^{14}C]GLUCOSAMINE INTO PRODUCTS NOT DIGESTIBLE BY LYSOZYME

Cells were labeled with N-benzoyl-[^{14}C]glucosamine. After 90 min, incorporation into hot TCA-insoluble fraction before and after digestion with lysozyme was determined. Incubation, determination of incorporation in the hot TCA-insoluble fraction and lysozyme digestion were performed as described in *2.2.13*, *2.2.14* and *2.2.17* respectively.

	Incorporation (dpm)	Incorporation after lysozyme treatment (dpm)	%
No antibiotic	16533	235	1.4
Chloramphenicol	7332	82	1.1
Tetracyclin	8601	35	0.4
Actinomycin-D	4301	34	0.8

It was found that the fraction of non-lysozyme-digestible, labeled material was not significant. This implies that nearly all the N-benzoyl-[^{14}C]glucosamine incorporated into the TCA-insoluble fraction is incorporated into the peptidoglycan.

2.3.9. Synthesis of lipids during inhibition of protein biosynthesis

In Fig. 11a the [^{14}C]oleic acid incorporation during inhibition of protein synthesis by chloramphenicol is shown. Whereas protein synthesis is stopped, synthesis of lipids continues significantly. This is also evident in the membrane lipid fraction (Fig. 11b), although the incorporation pattern shows a discrepancy with that found for the total lipids. The labeling rate for the total lipids is nearly constant whereas the labeling of membrane fraction stops after some time. The total ^{14}C-radioactivity incorporated in the total cell after

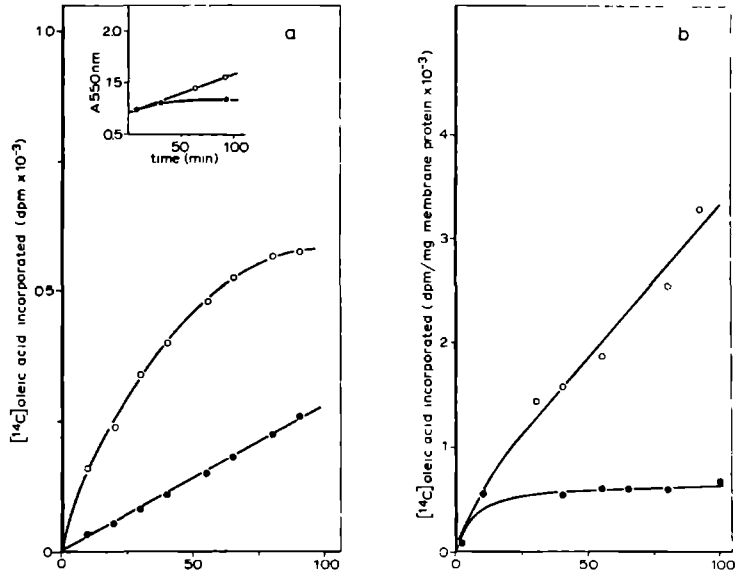

Fig. 11. Incorporation of oleic acid during inhibition with chloramphenicol. Cells were incubated with [^{14}C]oleic acid in the presence of 60 µg/ml chloramphenicol (closed symbols) and in the absence of chloramphenicol (open symbols). Incubation, measurement of incorporation, and determination of the bacterial density are described in MATERIALS AND METHODS (2.2).

a. Insertion: absorbance of the culture;
   Incorporation of [^{14}C]oleic acid into the cell;
b. Incorporation of [^{14}C]oleic acid into the membrane fraction per mg membrane protein.

90 min is 46% of the radioactivity in control cells.
In the presence of tetracyclin lipid synthesis in the total cell and in the cell membrane continued markedly (Fig. 12a and 12b respectively). From 0 to 60 min the rate of incorporation of [^{14}C]oleic acid in inhibited cells was 65% of that in the control cells. After 90 min incubation the radioactivity in the membrane fraction per mg membrane protein of inhibited cells was 80% of that in the normal cells. Actinomycin-D had a remarka e effect on oleic acid incorporation. From 0 to 60 min after the addition of [^{14}C]oleic acid the incorporation into the inhibited cells proceeded at about the same rate as for the control cells, but after 60 min the rate of incorporation was higher

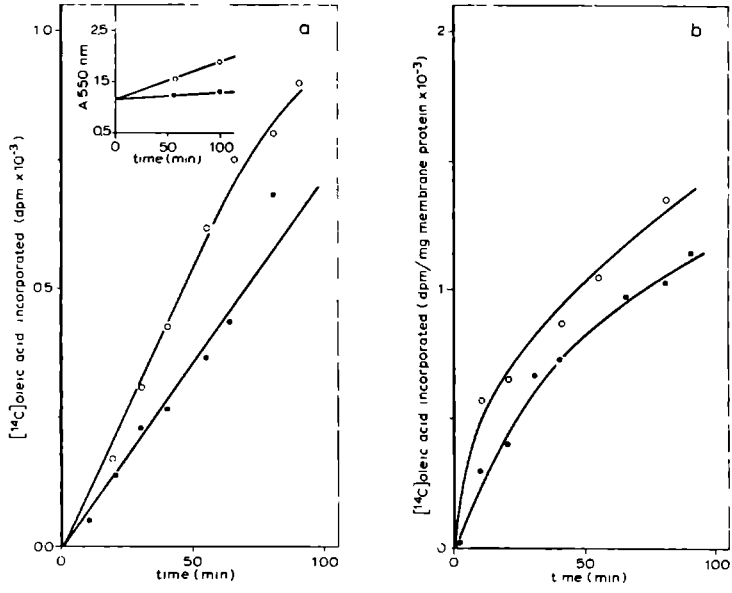

Fig. 12. Incorporation of oleic acid during inhibition with tetracyclin. Cells were incubated with [^{14}C]oleic acid in the presence of 50 µg/ml tetracyclin (closed symbols) and in the absence of tetracyclin (open symbols). Details are given in Fig. 11.

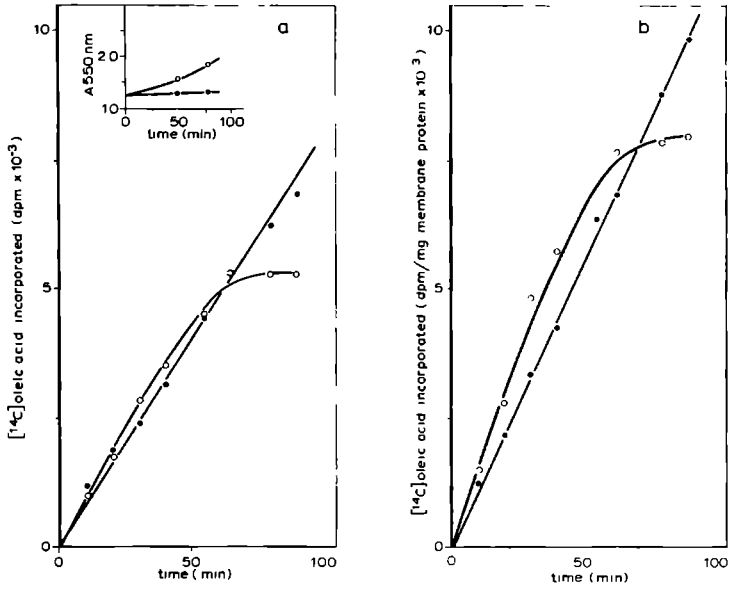

Fig. 13. Incorporation of oleic acid during inhibition with actinomycin-D. Cells were incubated with [^{14}C]oleic acid in the presence of 10 µg/ml actinomycin-D (closed symbols) and in the absence of actinomycin-D (open symbols). Details are given in Fig. 11.

than for the control cells (Fig. 13a). For the membrane fraction a similar incorporation pattern as for the total cell lipids was found (Fig. 13b).

Summarizing the results it was found that inhibition of protein biosynthesis did not arrest lipid synthesis in the total cell and in the membrane fraction. However, the effects on lipid synthesis were not the same for the various antibiotics. With the exception of inhibi-

tion by chloramphenicol, the results obtained for the lipid synthesis in total cells agree well with the results found for lipid synthesis in the membrane fraction. We shall discuss the results in the last section of this chapter.

2.3.10. Distribution of [^{14}C]oleic acid over different lipid fractions

In *2.3.9* it was shown that lipid synthesis continued in the absence of protein synthesis. For a correct interpretation of the results it was important to know whether the oleic acid was really incorporated

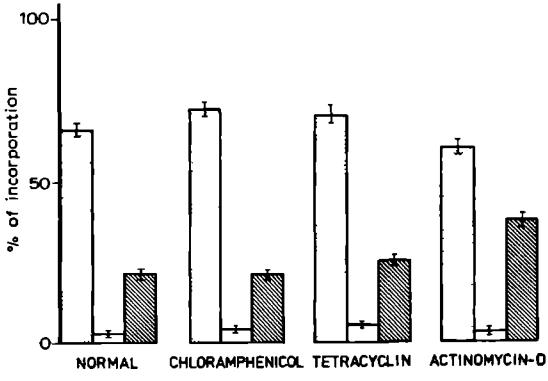

Fig. 14. Distribution of [^{14}C]oleic acid into lipid fractions of normal and inhibited cells in the presence of various antibiotics. Cells were incubated with [^{14}C]oleic acid during 60 min. Lipids were extracted and radioactivity in the lipid fractions was determined after thin layer silica gel chromatography. Incubation conditions, extraction of lipids and chromatographic procedures are described in *2.2.13*, *2.2.19* and *2.2.20* respectively.

1st bar: percentage of incorporation into the polar lipids;

2nd bar: percentage of incorporation into the free oleic acid fraction;

3rd bar: percentage of incorporation into the neutral lipids (without free oleic acid).

into the bacterial lipids or remained in the free fatty acid form. Incorporation of [^{14}C]oleic acids was therefore studied separately in the three groups of lipids: neutral lipids, free fatty acids and polar lipids (phospho- and glycolipids).
In normal cells 65% of the label was found in the polar lipids, 3% in the free fatty acid fraction and 33% in the neutral lipids. The relative distribution of radioactivity in the inhibited cells was not very different from that in normal cells (Fig. 14).
In normal and inhibited cells only a relatively small fraction of the incorporated [^{14}C]oleic acid occurs in the free fatty acid form.

2.3.11. Inhibition of peptidoglycan synthesis

Penicillin G was a strong inhibitor of growth. The inhibited culture did not rise much in absorbance while that of control culture increased by 100% (Fig. 15a). Almost no incorporation of N-benzoyl-[^{14}C]glucosamine into inhibited cells could be detected, whereas the control culture showed a significant incorporation. However, inhibition of cell growth with penicillin G also affected incorporation of [^{3}H]glycine into the cellular protein and the membrane protein. After 90 min of incubation the amount of [^{3}H]glycine incorporated into the cellular protein of the inhibited cells was only about 11% of that of the normal cells. The amount of [^{3}H]glycine per mg membrane protein in the membrane fraction was about 14% of that in the control cells.
Bacitracin was an effective inhibitor of growth (Fig. 16a). The increase in absorbance was 10% for the inhibited culture and 100% for the control culture after 90 min of incubation. Under these conditions the peptidoglycan synthesis was only a small fraction of the synthesis in control cells (Fig. 16b). After 90 min of incubation the incorporation into inhibited cells was only 4% of the incorporation into control cells.
Incorporation of [^{3}H]glycine was also affected (Fig. 16a). After 90 min incubation with glycine the amount of incorporation into inhibited cells was only 18% of that in control cells. A similar depression for glycine incorporation is found for the membrane fraction.

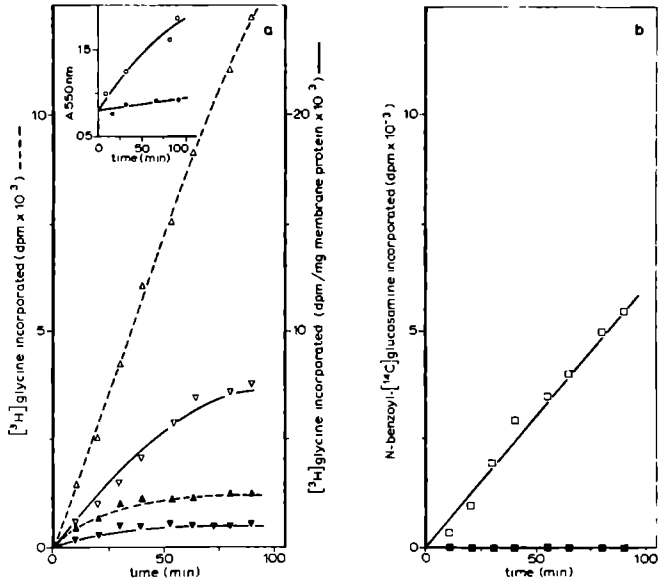

Fig. 15. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with penicillin G. Cells were incubated with radioactive precursors in the presence of 10 µg/ml penicillin G (closed symbols) and in the absence of penicillin G (open symbols). Details are given in Fig. 8.

These results are in agreement with those found for penicillin-inhibited cells.

D-cycloserine inhibited growth under our experimental conditions less than penicillin G and bacitracin. The increase in absorbance in the inhibited culture was 20% after 90 min incubation whereas the control culture increased about 80% in density (Fig. 17a). The effect on peptidoglycan synthesis is less pronounced than for penicillin and bacitracin inhibition (Fig. 17b). In the inhibited culture the incorporation of N-benzoyl-[^{14}C]glucosamine was 24% of that in the control cells after 90 min. The reason for this weaker inhibition may be in the fact that D-cycloserine is a competitive inhibitor of two enzymatic reactions in the synthesis of peptidoglycan. The

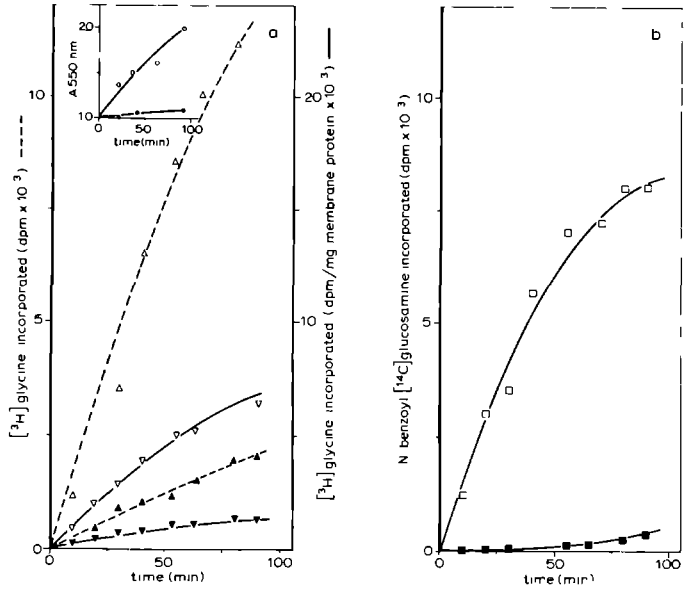

Fig. 16. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with bacitracin. Cells were incubated with radioactive precursors in the presence of 160 µg/ml bacitracin (closed symbols) and in the absence of bacitracin (open symbols). Details are given in Fig. 8.

inhibitory capacity of the inhibitor is in such a case dependent on the relative concentration of D-cycloserine and the competitive substrate (D-alanine). Inhibition of the enzymatic reactions is only complete when the D-cycloserine concentration in the cell may be considered infinitely large compared to that of D-alanine.
The influence on [^{3}H]glycine incorporation was also less marked than with penicillin G and bacitracin. After 90 min of incubation, incorporation into inhibited cells was 55% of that in the control culture. The incorporation into the membrane protein fraction per mg membrane protein was only slightly smaller in the inhibited cells than in the normal cells.
Vancomycin was an effective inhibitor of the bacterial growth. The

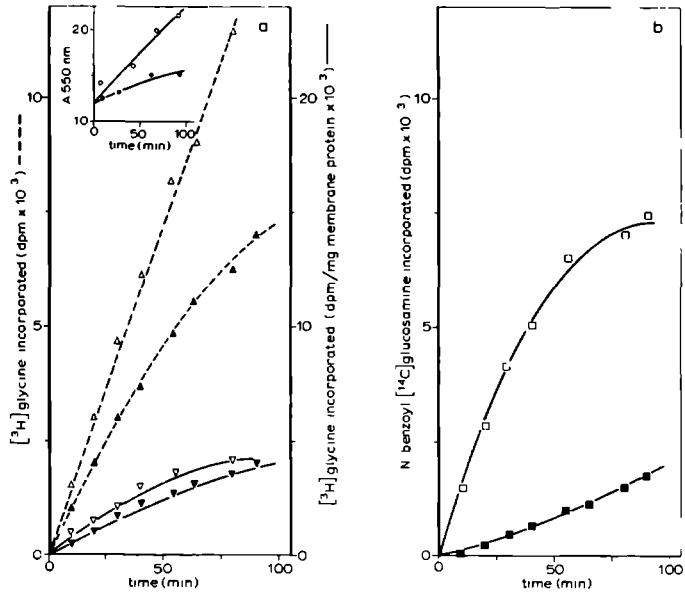

Fig. 17. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with D-cycloserine. Cells were incubated with radioactive precursors in the presence of 80 µg/ml D-cycloserine (closed symbols) and in the absence of D-cycloserine (open symbols). Details are given in Fig. 8.

absorbance of the inhibited culture remained at the same level during the entire incubation period (Fig. 18a). Incorporation of N-benzoyl-[^{14}C]glucosamine in the inhibited cells was not detectable (Fig. 18b). Again there is a concomitant effect on the incorporation of [^{3}H]glycine. Incorporation proceeded linearly in the inhibited culture and in the control culture but the rate of incorporation of the inhibited culture was only about 5% of that in the control culture. A decreased incorporation of [^{3}H]glycine in the membrane fraction per mg membrane protein was also found.

Omission of N-benzoyl-D-glucosamine from the culture medium resulted in inhibition of growth (Fig. 19a). The small increase of the bacterial density may be due to the intracellular presence of precursors

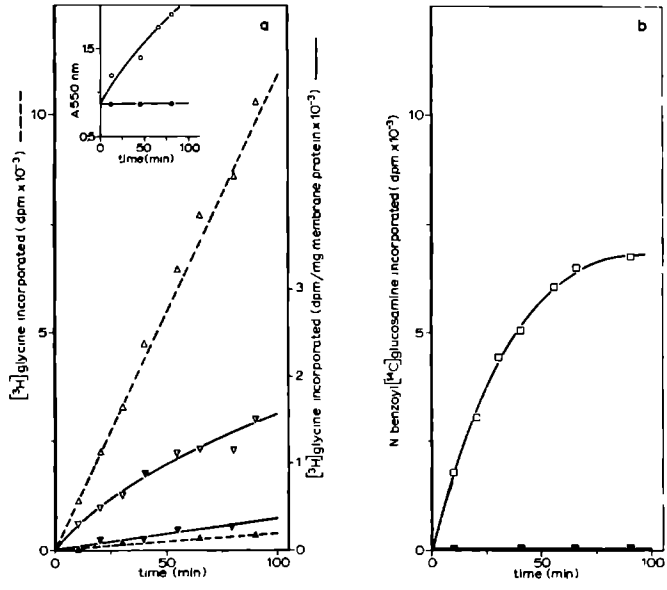

Fig. 18. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with vancomycin. Cells were incubated with radioactive precursors in the presence of 20 µg/ml vancomycin (closed symbols) and in the absence of vancomycin (open symbols). Details are given in Fig. 8.

for cell wall synthesis, which were present before deprivation of the culture. These precursors can allow cell wall synthesis until the pool of precursors is exhausted. [^{3}H]glycine incorporation in the inhibited cells is lower than in the control cells (Fig. 19a). In the linear sections of the plots, the rate of incorporation in the inhibited cells is about 51% of that in the control cells. The rate of incorporation per mg membrane protein in the inhibited cells is also about 50% of that in the control culture (Fig. 19b). Fig. 20a shows the absorbance of the culture inhibited by staphylococcin and the control culture. A small increase of density is apparent in the inhibited culture during incubation (about 10%) compared with an increase of 150% in the control culture.

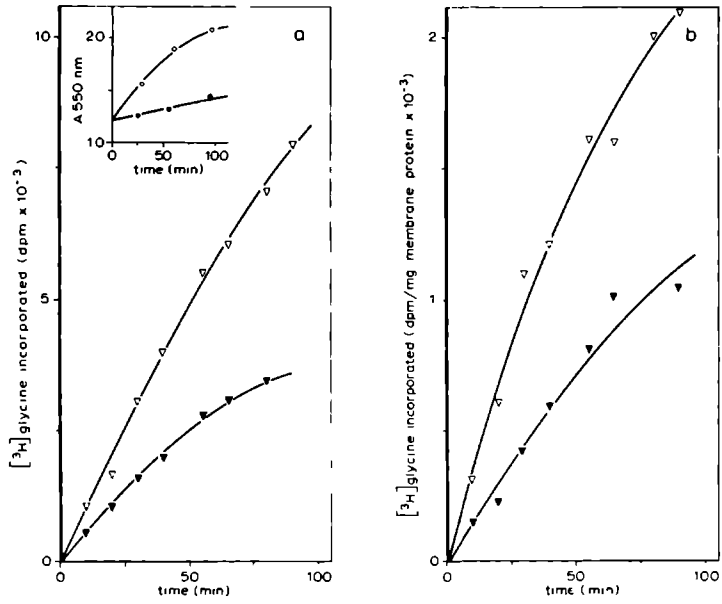

Fig. 19. Incorporation of glycine during omission of N-benzoyl-glucosamine. Cells were incubated with radioactive precursors in the presence of 25 µg/ml N-benzoyl-glucosamine (open symbols) and in the absence of N-benzoyl-glucosamine (closed symbols). Details are given in Fig. 8.

The incorporation of N-benzoyl-[^{14}C]glucosamine in the inhibited culture proceeded linearly but was much depressed compared to the control culture (Fig. 20b). The incorporation in the inhibited cells was (in the linear parts of the curves) only 7% of that in the normal cells. Concomitant with the inhibition of growth an inhibition in the incorporation of [^{3}H]glycine was observed. After an incubation period of 90 min the incorporation of glycine in the inhibited cells was only 17% of that in the control cells. The incorporation of glycine into the membrane fraction per mg membrane protein was about 25% of that in the control culture after 90 min of incubation.

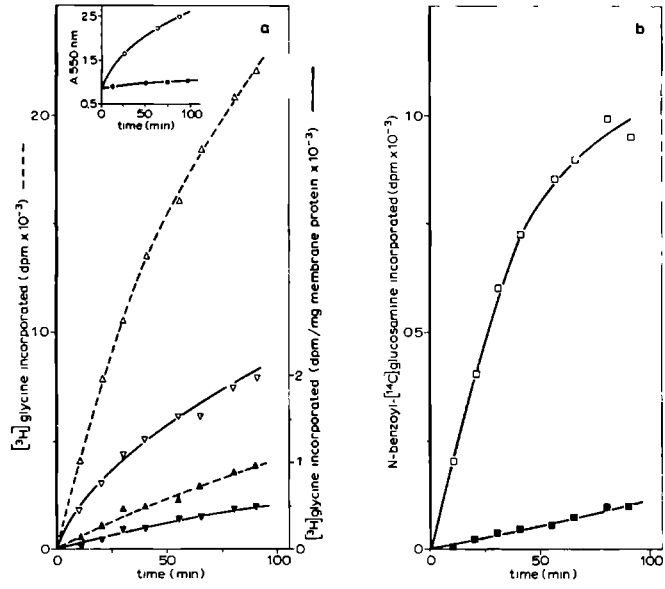

Fig. 20. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during inhibition with staphylococcin 1580. Cells were incubated with radioactive precursors in the presence of 50 A.U/ml staphylococcin (closed symbols) and in the absence of staphylococcin (open symbols). Details are given in Fig. 8.

2.3.12. *Incorporation of [^{14}C]oleic acid during inhibition of peptidoglycan synthesis*

Penicillin G had a remarkable effect on the incorporation of [^{14}C]oleic acid into the cells (Fig. 21a). The inhibited culture increased less in absorbance compared with the increase in the control culture, after 90 min incubation. Control cells showed again an increase of the amount of oleic acid incorporated, followed by a decline. The incorporation in inhibited cells took place with a relatively high rate and was significantly higher than in the control cells. The incorporation reached a maximum at about 30 min after adding the oleic acid and decreased thereafter. The amount of oleic acid

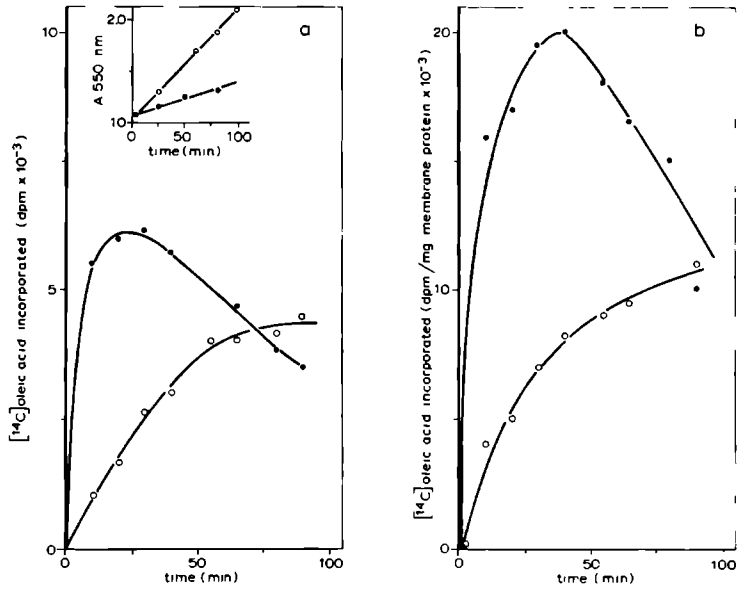

Fig. 21. Incorporation of oleic acid during inhibition with penicillin G. Cells were incubated with [^{14}C]oleic acid in the presence of 10 µg/ml penicillin G (closed symbols) and in the absence of penicillin G (open symbols). Details are given in Fig. 11.

incorporated into the membrane fraction, expressed on the basis of membrane protein content, showed an identical pattern: an initially higher incorporation rate than in the normal cells, a maximum value and a decrease of label per mg membrane protein.

The culture inhibited by bacitracin increased not much in absorbance compared to the control culture after 90 min incubation (Fig. 22). Bacitracin had no marked effect on the [^{14}C]oleic acid incorporation from 0 to 25 min after addition of the precursor to the cells. However, after 25 min the incorporation curve for the control cells declined, while the incorporation in the inhibited cells continued to increase. After 90 min the incorporation into the inhibited cells was 170% of that into control cells. The incorporation into the mem-

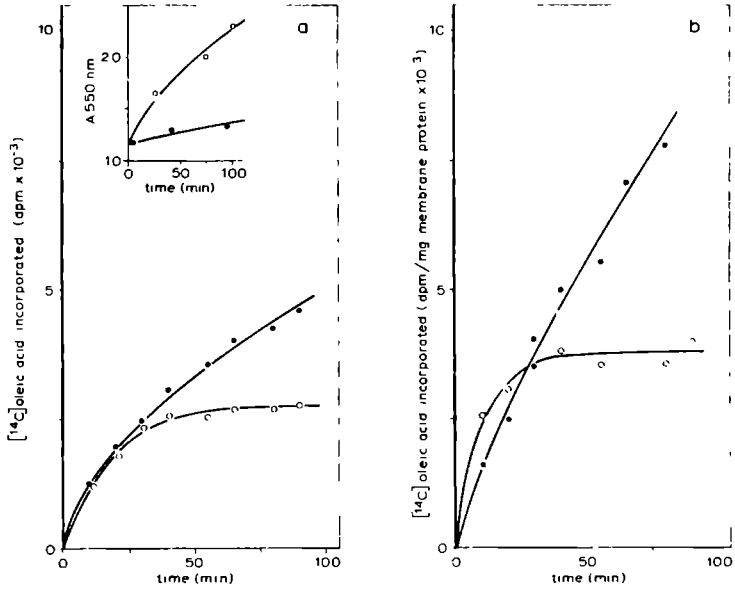

Fig. 22. Incorporation of oleic acid during inhibition with bacitracin. Cells were incubated with [^{14}C]oleic acid in the presence of 160 µg/ml bacitracin (closed symbols) and in the absence of bacitracin (open symbols). Details are given in Fig. 11.

brane fraction showed an almost identical incorporation pattern (Fig. 22b).

The culture inhibited by D-cycloserine, showed an increase of absorbance of 30% during 90 min incubation, compared with a 66% increase in the control culture (Fig. 23). The incorporation of [^{14}C]oleic acid into the inhibited cells showed a higher incorporation rate than that into control cells. After 90 min of incubation the amount of oleic acid incorporated was 144% of that in the control cells. The incorporation into the membrane fraction showed an identical pattern as the incorporation curves for oleic acid in total cells (Fig. 23b).

The culture inhibited by vancomycin increased about 10% and the con-

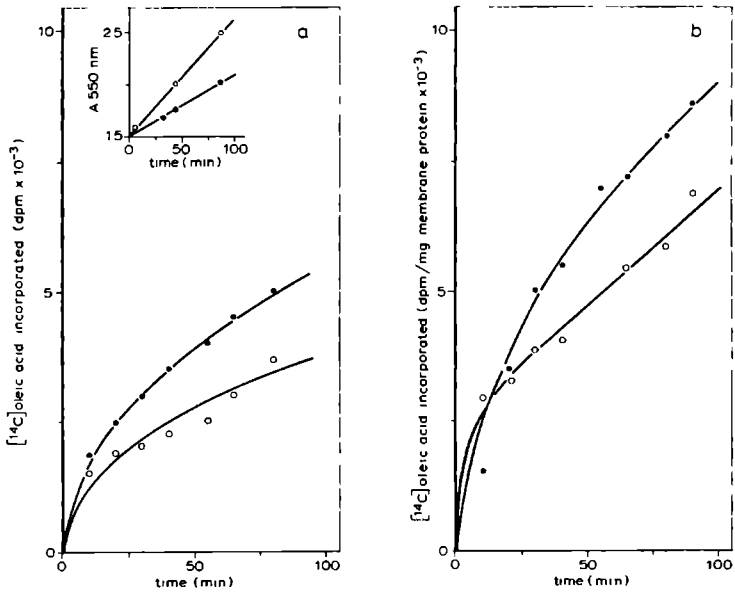

Fig. 23. Incorporation of oleic acid during inhibition with D-cycloserine. Cells were incubated with [^{14}C]oleic acid in the presence of 80 µg/ml D-cycloserine (closed symbols) and in the absence of D-cycloserine (open symbols). Details are given in Fig. 11.

trol culture about 90% in absorbance (Fig. 24a) during the incubation. The incorporation of [^{14}C]oleic acid showed a pattern which is very similar to that found for penicillin G-treated cells. The incorporation of oleic acid into the inhibited cells is higher than into the control cells during the entire incubation period. Incorporation into inhibited cells showed a maximum about 20 min after addition of the oleic acid and then decreased. The incorporation pattern of oleic acid into the membrane agreed with that found for the incorporation into the total cell (Fig. 24b).

Omission of N-benzoyl-glucosamine inhibited the bacterial growth. The deprived culture increased only about 8% whereas the control culture increased about 65% in absorbance (Fig. 25). Under these conditions

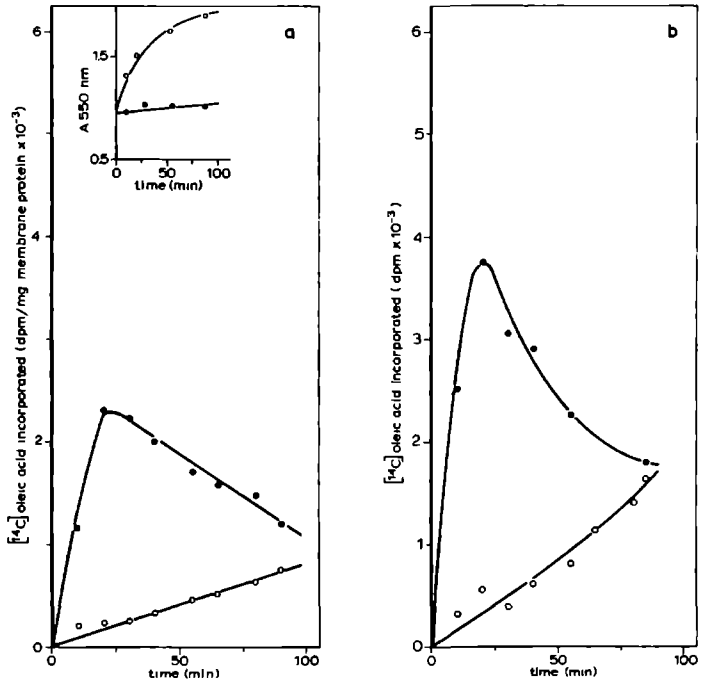

Fig. 24. Incorporation of oleic acid during inhibition with vancomycin. Cells were incubated with [^{14}C]oleic acid in the presence of 20 µg/ml vancomycin (closed symbols) and in the absence of vancomycin (open symbols). Details are given in Fig. 11.

the incorporation of [^{14}C]oleic acid into the inhibited cells was only slightly smaller than that in the control cells from 0 to 25 min after addition of the precursor. After 25 min the incorporation became significantly higher than in the control cells. After 90 min incubation the amount of oleic acid incorporated into the inhibited cells was about 127% of that in the control cells.

Whereas the control culture increased about 150%, the culture inhibited by staphylococcin increased only 20% in absorbance (Fig. 26a). Incorporation of [^{14}C]oleic acid into inhibited cells was higher than in control cells from 0 to 40 min after addition of the oleic acid; the incorporation curve then clearly declined and remained below the

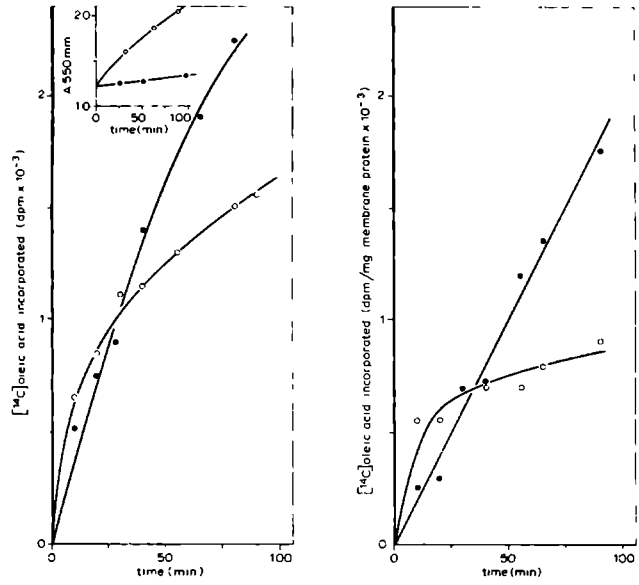

Fig. 25. Incorporation of oleic acid during omission of N-benzoyl-glucosamine. Cells were incubated with [^{14}C]oleic acid in the presence of 25 µg/ml N-benzoyl-glucosamine (open symbols) and in the absence of N-benzoyl-glucosamine (closed symbols). Details are given in Fig. 11.

incorporation plot for control cells.
The incorporation of [^{14}C]oleic acid per mg membrane protein was higher in the inhibited cells than in the normal cells during the entire incubation period (Fig. 26b).

2.3.13. Distribution of [^{14}C]oleic acid over different lipid fractions

During inhibition of cell wall peptidoglycan synthesis in bacteria the incorporation of [^{14}C]oleic acid into cells was markedly increased in comparison to normal cells. Since this was an interesting phenomenon, we investigated whether the increase of incorporation of label was

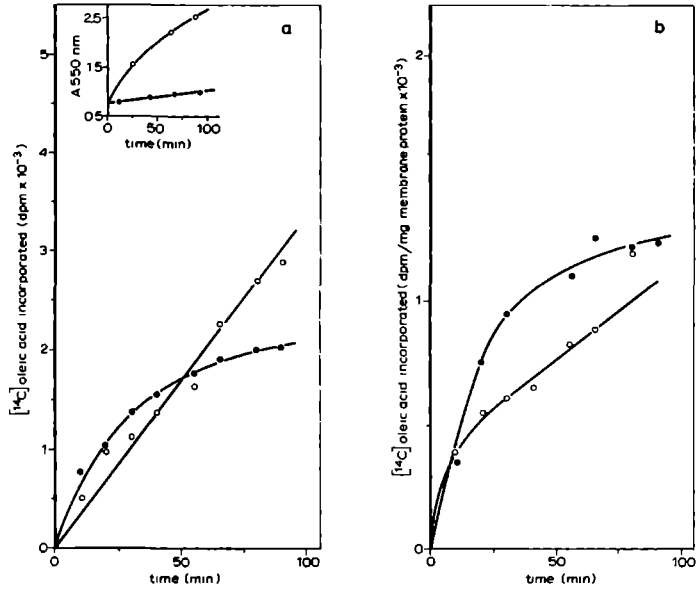

Fig. 26. Incorporation of oleic acid during inhibition with staphylococcin 1580. Cells were incubated with [^{14}C]oleic acid in the presence of 50 A.U./ml staphylococcin (closed symbols) and in the absence of staphylococcin (open symbols). Details are given in Fig. 11.

due to incorporation into free fatty acids and neutral lipids. We studied the incorporation of oleic acid into the three lipid groups: neutral lipids, free fatty acids and polar lipids (glyco- and phospholipids). No significant differences in the distribution of the label over the lipid fractions were detected between normal and inhibited cells (Fig. 27). There was only a relatively small amount of [^{14}C]oleic acid in the free fatty acid form (about 5%). This indicates that the increased incorporation of [^{14}C]oleic acid into the inhibited cells is due to an increase in its incorporation in neutral and membrane lipids.

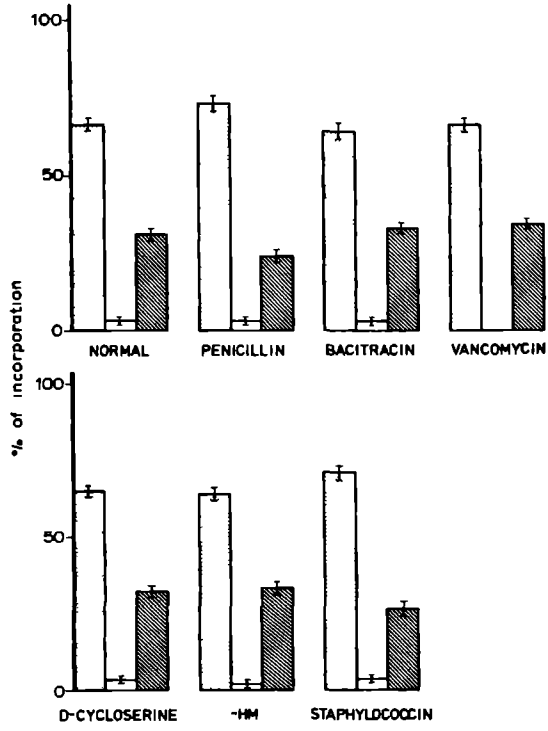

Fig. 27. Distribution of [^{14}C]oleic acid into lipid fractions of normal and inhibited cells. Cells were incubated with [^{14}C]oleic acid during 60 min. Lipids were extracted and radioactivity in the lipid fractions was determined after thin-layer silica gel chromatography. Incubation conditions, extraction of lipids and chromatographic procedures are described in *2.2.13*, *2.2.19* and *2.2.20* respectively.
1st bar: percentage of incorporation into the polar lipid;
2nd bar: percentage of incorporation into the free oleic acid fraction;
3rd bar: percentage of incorporation into the neutral lipid (without free oleic acid).

2.3.14. *Incorporation of [^{14}C]palmitic acid during inhibition of cell wall biosynthesis*

In the preceding section it was shown that during inhibition of cell wall biosynthesis incorporation of [^{14}C]oleic acid generally increased in the inhibited cells as compared to normal cells. In this section the influence of inhibition of cell wall biosynthesis on the incorporation of palmitic acid is studied.
The influence of penicillin G, bacitracin, vancomycin, D-cycloserine, omission of growth factor and staphylococcin 1580 is shown in Fig. 28. Penicillin G increased palmitic acid incorporation from 0 to 50 min after addition of this precursor. After about 40 min a maximum value was reached and thereafter the amount of [^{14}C]palmitic acid incorporated decreased. Bacterial growth was almost fully inhibited in this experiment. Cells inhibited by bacitracin incorporated more palmitic acid than control cells between 0 and 70 min of incubation. After 70 min incubation incorporation into the control cells was higher than into the inhibited cells. Bacterial growth was nearly completely inhibited. Vancomycin stimulated incorporation of palmitic acid. Incorporation was higher in inhibited cells than in control cells during the entire incubation period. However, after about 60 min a maximum in the incorporation was reached whereupon the incorporation decreased. In this experiment bacterial growth was not optimally inhibited since the inhibited culture increased its absorbance by 40% of that of the control culture. It is clear from Fig. 28 that the incorporation of palmitic acid is significantly increased in the presence of D-cycloserine, staphylococcin and by depletion of N-benzoyl-glucosamine. The incorporation of palmitic acid in cells inhibited by D-cycloserine and by staphylococcin 1580 tended to decline at the end of the incubation period.

2.3.15. *Distribution of [^{14}C]palmitic acid over different lipid fractions*

The distribution of [^{14}C]palmitic acid over the lipids of inhibited labeled cells was determined. The same methods were used as described in *2.3.10*. The distribution of [^{14}C]palmitic acid in control cells

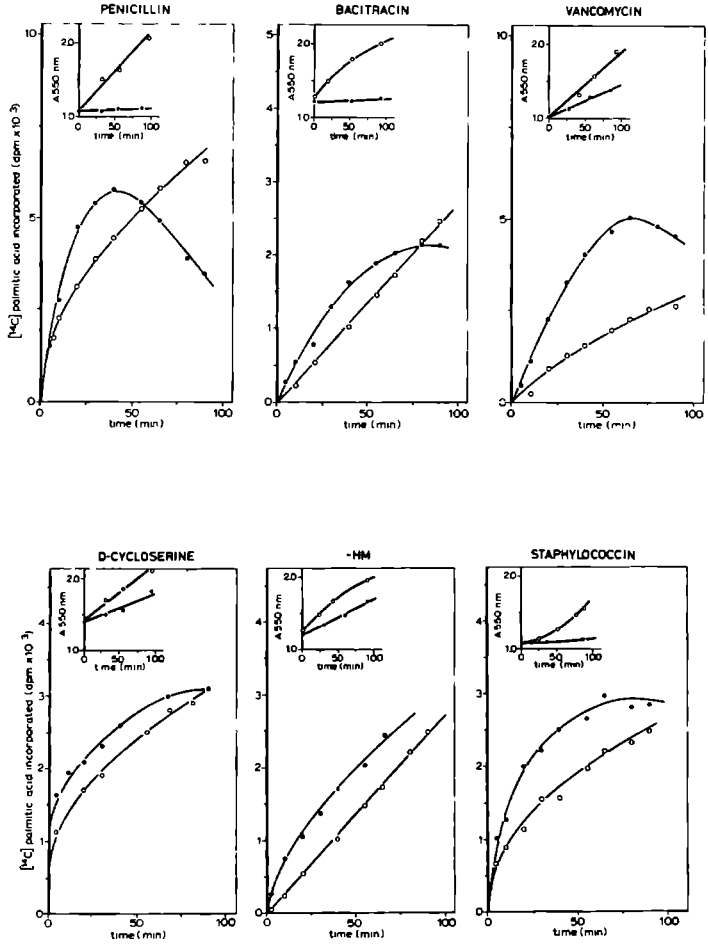

Fig. 28. Incorporation of palmitic acid during inhibition of peptidoglycan synthesis by various antibiotics. Cells were incubated with [^{14}C]palmitic acid in the presence of different inhibitors and in the absence of N-benzoyl-glucosamine. Results for inhibited cells are represented with closed symbols and for control cells with open symbols. Insertion: absorbance of the cultures. Incubation conditions, measurement of incorporation and determination of absorbance are described in MATERIALS AND METHODS (2.2).

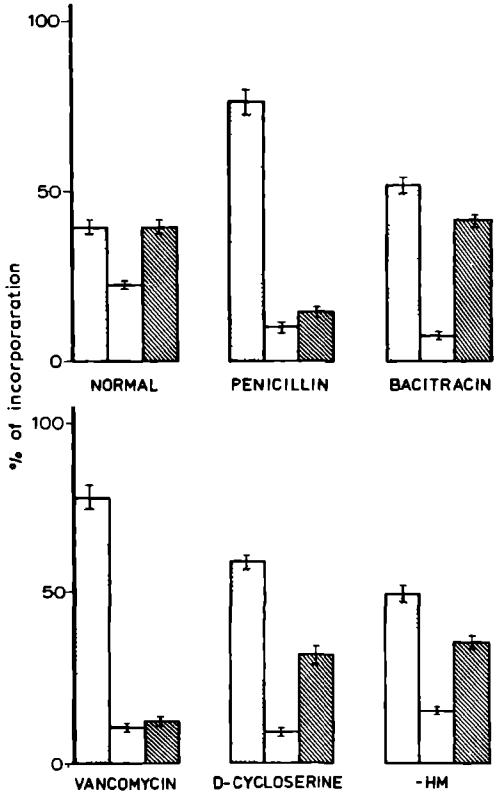

Fig. 29. Distribution of [^{14}C]palmitic acid into lipid fractions of normal and inhibited cells. Cells were incubated with [^{14}C]palmitic acid during 60 min. Lipids were extracted and radioactivity in the lipid fraction was determined after thin-layer silica gel chromatography. Incubation conditions, extraction of lipids and chromatographic procedures are described in *2.2.13*, *2.2.19* and *2.2.20* respectively.
1st bar: percentage of incorporation into the polar lipids;
2nd bar: percentage of incorporation into the free palmitic acid fraction;
3rd bar: percentage of incorporation into neutral lipids (without free palmitic acid).

(Fig. 29) showed a different pattern than for [^{14}C]oleic acid labeled cells (*2.3.13*). Especially the fraction of free palmitic acid was relatively high compared to the free oleic acid fraction found in cells, labeled with [^{14}C]oleic acid. However, the fraction of free palmitic acid in inhibited cells was lower than in normal cells. During inhibition, the relative incorporation into the polar lipids was increased as compared to control cells. These different incorporation patterns make a comparison of the incorporation into inhibited cells and normal cells more complicated. Comparison of the incorporation of [^{14}C]palmitic acid into the inhibited cells would be relatively higher, if only the polar lipid fractions were compared. Glycolipids and phospholipids are characteristic lipids for the membrane. This suggests that the incorporation of [^{14}C]palmitic acid into the membrane is probably more increased than is apparent from the data for the total cells (Fig. 28).

2.3.16. Influence of omission of sodium acetate and Mg^{2+} on the incorporation of [^{3}H]glycine and N-benzoyl-[^{14}C]glucosamine

The rate of incorporation of [^{3}H]glycine into normal and inhibited cells was about the same from 0 to 10 min, but thereupon the incorporation decreased for inhibited cells. The labeling in the membrane fraction was depressed compared to that of the normal cells (Fig. 30). These data show an important effect on the incorporation of [^{3}H]glycine and indicate an inhibition of protein synthesis due to sodium acetate omission. An inhibition of incorporation of N-benzoyl-[^{14}C]glucosamine was also apparent. Between 0 and 60 min the rate of incorporation in inhibited cells was 54% of that in control cells. Omission of $MgSO_4$ from the culture medium resulted in a decreased Mg^{2+} concentration from 0.80 to 0.14 mMol/l. The effects on the organism of growth in this Mg^{2+}-poor medium are not predictable since:

(i) some cellular reactions need only small amounts of Mg^{2+};

(ii) despite reduction of the Mg^{2+} content in the culture medium a localized high Mg^{2+} concentration may be present intracellularly;

(iii) despite reduction of the Mg^{2+} content in the culture medium the

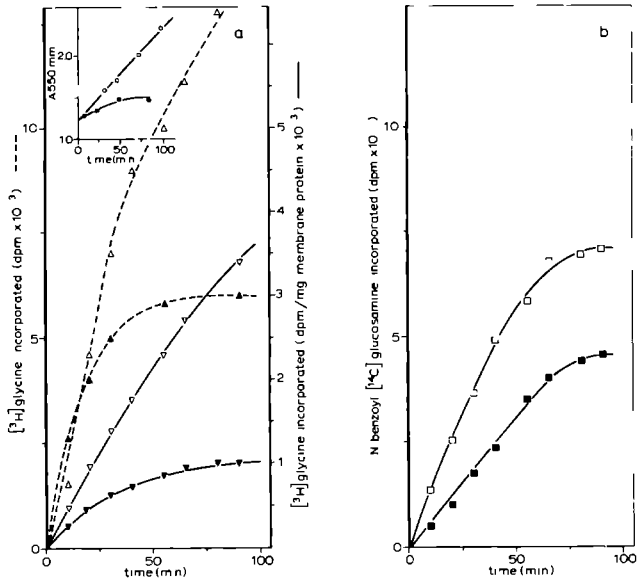

Fig. 30. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during omission of sodium acetate. Cells were incubated with radioactive precursors in the normal medium (open symbols) and in a medium from which sodium acetate was omitted (closed symbols). Details are given in Fig. 8.

original cells have a certain amount of Mg^{2+} ions intracellularly.

The growth rate of the Mg^{2+}-poor culture was reduced to 40% of that in the control culture (Fig. 31a). The incorporation of $[^3H]$glycine into the total cellular protein was less than in normal cells. From 0 to 40 min the incorporation into inhibited cells and control cells proceeded about linearly. The rate of incorporation into inhibited cells was 62% of that into normal cells. The incorporation of glycine into the membrane fraction was also lower in the inhibited cells than for the control cells. In the linear sections of the incorporation curves the incorporation rate for inhibited cells was about 66%

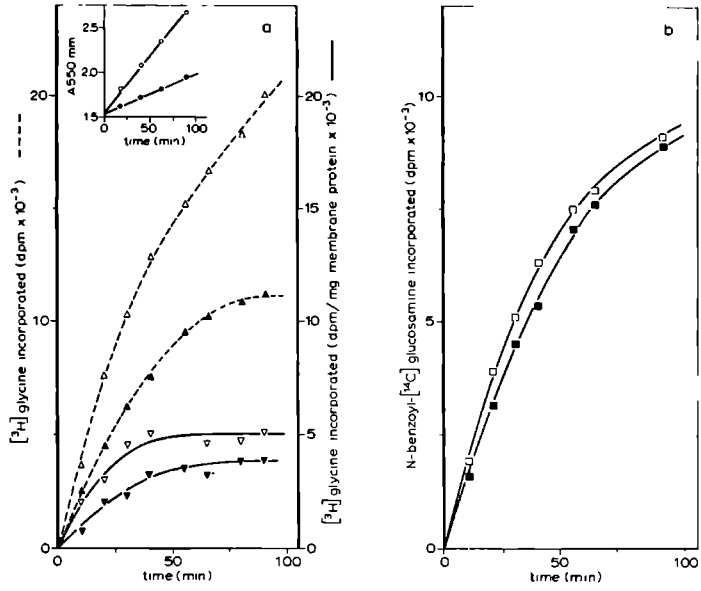

Fig. 31. Incorporation of glycine (a) and N-benzoyl-glucosamine (b) during growth in Mg^{2+}-poor medium. Cells were incubated with radioactive precursors in normal medium (open symbols) and in Mg^{2+}-poor medium (closed symbols). Details are given in Fig. 8.

of that for the control cells.
Incorporation of N-benzoyl-[^{14}C]glucosamine into inhibited cells and control cells increased from 0 to 50 min and then tended to decline. Incorporation into inhibited cells and into control cells did not differ much. The incorporation rate into inhibited cells was in the linear parts of the incorporation curves 90% of that in the control cells despite the much lower density of the inhibited culture (Fig. 31b).
During omission of sodium acetate and Mg^{2+} from the culture medium there was a significant incorporation of N-benzoyl-[^{14}C]glucosamine into the hot TCA-insoluble fraction of the cells.
By the lysozyme digestion procedure (*2.2.17*) we determined the amount of N-benzoyl-[^{14}C]glucosamine incorporated into products insoluble in hot TCA but indigestible by lysozyme.

As we observed before (*2.3.1*), only 1.4% of the incorporated radioactivity in normal cells was not lysozyme-digestible. After omission of Mg^{2+} from the culture medium only 1.3% of the radioactivity incorporated after 60 min incubation was not digestible by lysozyme. However, after omission of sodium acetate from the culture medium, the amount of radioactivity present in the hot TCA-insoluble fraction of cells labeled during 60 min was increased to 8.5%. This indicates that 8.5% of the radioactivity is incroporated into lysozyme-resistant products. We also found other indications for lysozyme-resistance in cells grown in the absence of sodium acetate. Cells cultivated for 16 h in media without sodium acetate appeared to be lysozyme-resistant. Microscopic observation showed no morphological differences in the cell shape after incubation of these cells with lysozyme (1 mg/ml) during 40 to 90 min, whereas the protoplasts of the normal cells (after 16 h growth) changed from the rod shape to the spherical shape.

2.3.17. Influence of omission of sodium acetate and Mg^{2+} on the incorporation of oleic acid

During sodium acetate omission (Fig. 32) incorporation of [^{14}C]oleic acid into cells was slightly smaller than into control cells from 0 to 50 min. The incorporation into inhibited cells proceeded linearly. The incorporation into the membrane fraction became higher after 30 min compared to control cells. It is important to note that, whereas incorporation of [^{3}H]glycine into the membrane fraction increased only slowly, there was a rapid linear increase in incorporation of [^{14}C]oleic acid.

During Mg^{2+} omission of cells (Fig. 33) there was a significantly lower incorporation of [^{14}C]oleic acid into inhibited cells than for control cells. If the incorporation rates from 0 to 40 min are compared, the rate of incorporation into inhibited cells is only 38% of that of the control cells. This situation existed also for incorporation into the membrane fraction. From 0 to 40 min the rate of incorporation was 32% of that for the control cells.

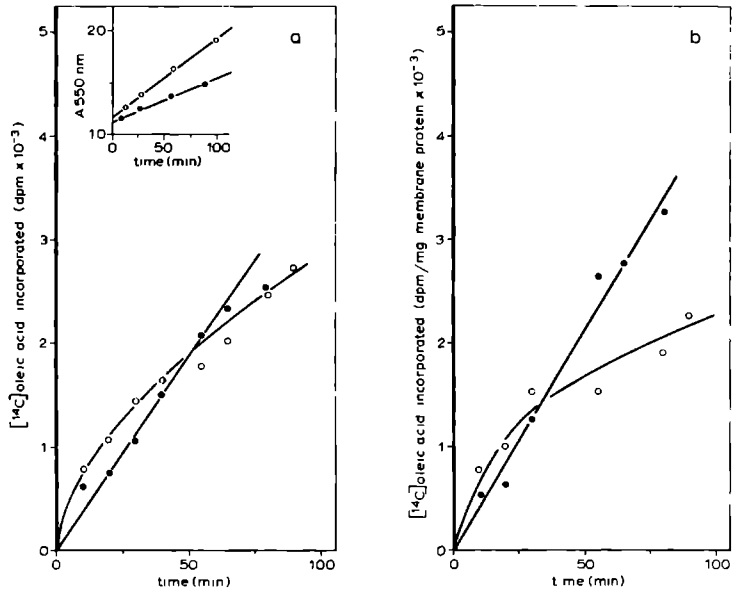

Fig. 32. Incorporation of oleic acid during omission of sodium acetate. Cells were incubated with [^{14}C]oleic acid in the normal medium (open symbols) and in a medium from which sodium acetate was omitted (closed symbols). Details are given in Fig. 11.

2.3.18. Distribution of [^{14}C]oleic acid over different lipid fractions

Incorporation of [^{14}C]oleic acid in neutral lipids, free fatty acids, glycolipids and phospholipids was studied. The results for cells incubated for 60 min with [^{14}C]oleic acid show no significant difference in distribution over the different lipid fractions between normal cells and inhibited cells (Fig. 34).

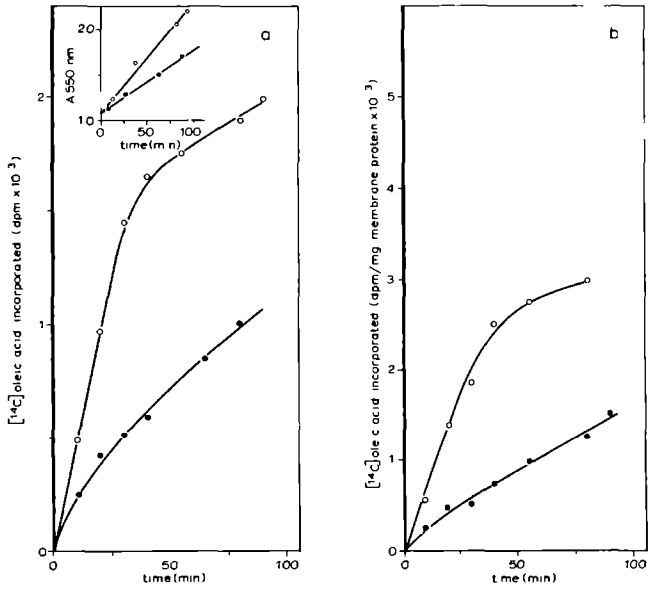

Fig. 33. Incorporation of oleic acid during growth in Mg^{2+}-poor medium. Cells were incubated with [^{14}C]oleic acid in normal medium (open symbols) and in Mg^{2+}-poor medium (closed symbols). Details are given in Fig. 11.

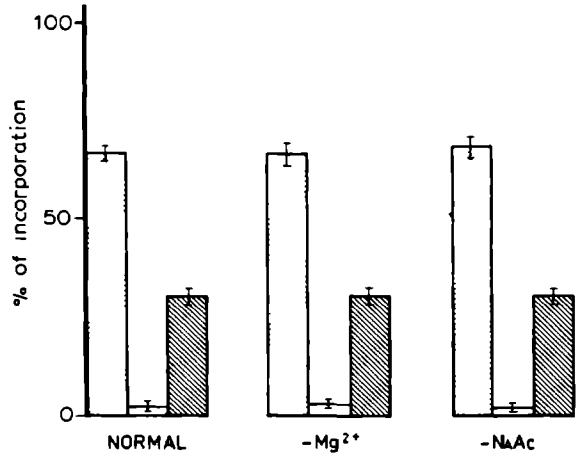

Fig. 34. Distribution of [^{14}C]oleic acid into lipid fractions of normal and inhibited cells. Cells were incubated with [^{14}C]oleic acid during 60 min. Lipids were extracted and radioactivity in the lipid fractions was determined after thin-layer silica gel chromatography. Incubation conditions, extraction of lipids and chromatographic procedures are described in *2.2.13*, *2.2.19* and *2.2.20* respectively.
1st bar: percentage of incorporation into polar lipids;
2nd bar: percentage of incorporation into the free oleic acid fraction;
3rd bar: percentage of incorporation into the neutral lipids (without free oleic acid).

2.4. DISCUSSION

In the preceding section we described how synthetic processes in cell wall and cell membrane were studied under conditions of normal and inhibited growth.
N-benzoyl-D-glucosamine, glycine and oleic acid appeared to be suitable precursors for cell wall peptidoglycan, cellular protein and cellular lipids respectively. A membrane isolation technique was applied to separate the membrane fraction from the other cellular constituents. Incorporation of labeled precursors into cells of the exponential growth phase was studied during a relatively short incubation time (3 h). A survey of characteristic results is given in Table VII. Under our experimental conditions, chloramphenicol, tetracyclin and actinomycin-D inhibited protein synthesis almost completely. An interesting finding was that peptidoglycan synthesis continued to a considerable extent. However, the rate of peptidoglycan synthesis in cells inhibited by actinomycin-D, was lower than in cells inhibited by chloramphenicol and tetracyclin. Possibly this is due to a concomitant effect of actinomycin-D on peptidoglycan synthesis. The lysozyme digestion experiments proved that N-benzoyl-glucosamine was really incorporated into peptidoglycan material.

There is only one study (Wong et al., 1974) on the effect of antibiotics on cell wall peptidoglycan synthesis in which a carbohydrate precursor was reported. In other studies peptidoglycan synthesis was investigated by incorporation of amino acids (Hancock and Park, 1958; Mandelstam and Rogers, 1959; Kitanaka et al., 1972; Wilkinson, 1973). Hancock and Park (1958) found that chloramphenicol did not affect lysine, alanine and glutamic acid incorporation into the peptidoglycan of *S. aureus* during short incubation times. Mandelstam and Rogers (1959) found that *S. aureus*, incubated in the presence of glycine, glutamic acid and alanine, incorporated these amino acids almost exclusively into the cell wall peptidoglycan. This process was resistant to chloramphenicol, but sensitive to penicillin and bacitracin. Kitanaka et al. (1972) showed that increasing concentrations of inhibitors of protein synthesis (clindamycin, spiramycin and

TABLE VII. INFLUENCE OF ANTIBIOTICS AND NUTRIENT DEPLETION ON GROWTH AND INCORPORATION OF GLYCINE N-BENZOYL-GLUCOSAMINE AND OLEIC ACID

Data are calculated from the experiments described in 2.3 after 50 min incubation with the radioactive precursors. Values for normal cells were set at 100.

	Average growth	Relative incorporation				
		Glycine		N-benzoyl-glucosamine	Oleic acid	
		cells	membrane		cells	membrane
Normal 3 h	100	100	100	100	100	100
Chloramphenicol	27	5	1	50	32	14
Tetracyclin	23	2	2	47	65	79
Actinomycin-D	11	3	3	24	93	83
Penicillin G	21	13	18	1	129	209
Vancomycin	4	5	18	1	420	270
Bacitracin	13	16	14	2	122	155
Cycloserine	45	63	75	13	147	133
Without N-benzoyl-glucosamine	18	51	48	--	131	141
Staphylococcin	11	15	20	7	100	144
Without Mg^{2+}	42	62	66	90	38	32
Without sodium acetate	43	55	36	54	100	126

chloramphenicol) decreased incorporation of lysine into cellular protein and increased it into the cell wall peptidoglycan fraction of *S. aureus*. Wilkinson (1973) studied the effect of inhibitors of protein biosynthesis and peptidoglycan synthesis in *Pediococcus cerevisae*. Peptidoglycan synthesis, measured by incorporation of labeled alanine, glutamic acid and lysine, was not affected by chloramphenicol, but penicillin inhibited its biosynthesis. Wong et al. (1974) found that labeled N-acetyl-glucosamine was readily incorporated by *S. aureus*. More than 95% of the radioacitivity was recovered in the cell walls. The incorporation of N-acetyl-glucosamine was sensitive to penicillin G, D-cycloserine and vancomycin, but insensitive to chloramphenicol.
These results agree with our observations. We cannot relate our observations directly to a net synthesis of peptidoglycan under conditions of inhibition of protein synthesis at this place, but will come back on this point in Chapter 3.

Whereas membrane protein synthesis was almost completely inhibited, synthesis of lipids in the membrane continued, although at different rates for different inhibitors. There was good agreement between the data found for total cellular lipid synthesis and membrane lipid synthesis, with the exception of chloramphenicol inhibition where incorporation in the membrane was more depressed than cellular incorporation. The extent of oleic acid incorporation in actinomycin-D inhibited cells was relatively high. Despite the relatively small increase in absorbance the incorporation in inhibited cells was of the same rate as in control cells and even exceeded that of control cells after longer incubation times. The results are similar to those obtained by Kahane and Razin (1968) in analogous experiments. When protein synthesis was arrested by chloramphenicol in *Mycoplasma laidlawii* lipid synthesis was not affected. Our observations correspond also with other experiments which suggested that there is evidently no tight coupling between membrane protein and membrane lipid synthesis (Henning et al., 1969; Mindich, 1970; Ray et al., 1973; Goldberg et al., 1973). However, our observations for lipid synthesis cannot be related directly to net lipid synthesis.
During inhibition of protein synthesis, incorporation did not probably

occur preferentially in a single lipid fraction such as neutral lipids, since there were no significant differences in the distribution of radioactivity in the lipids of normal and inhibited cells. There may, however, exist differences in synthetic rates in the lipid fractions examined for the various lipid components. This point will be studied in Chapter 3.

Penicillin G, vancomycin, bacitracin, D-cycloserine and staphylococcin greatly reduced or prevented incorporation of N-benzoyl-glucosamine into the cell. This is an indication that under the experimental conditions, biosynthesis of peptidoglycan was inhibited. Possibly the inhibition of the peptidoglycan synthesis in *B. bifidum* var. *pennsylvanicus* by staphylococcin is an indirect consequence of its action. In all cases where inhibition of peptidoglycan biosynthesis was observed, the incorporation of [^{14}C]oleic acid into the cells was higher than in normal cells. This phenomenon was also observed when peptidoglycan synthesis was inhibited by omission of N-benzoyl-glucosamine and by action of actinomycin-D. The increased oleic acid incorporation was not only apparent in the total lipids but also in the membrane fraction. The increased incorporation was not due to an increased uptake into the free oleic acid pool or into the neutral lipids. During inhibition by penicillin G and vancomycin the incorporation of [^{14}C]oleic acid was strongly increased compared to control cells but after some time the amount of oleic acid incorporated decreased. This decrease may indicate that labeled lipids or degradation products of lipids leave the cell. This implies that leakage of lipids or their derivatives from the cells begins to dominate over the incorporation of the fatty acid into the lipids. Theoretically this could be due to:

1. Increasing elimination of [^{14}C]oleic acid compared to the incorporation of [^{14}C]oleic acid;
2. Decreasing incorporation of [^{14}C]oleic acid compared to an elimination process already operating during the incubation;
3. A combination of both possibilities.

The phenomena found with vancomycin and penicillin may be caused by a secondary action of these antibiotics or they may be due to the possibility that membrane damage occurs by being not well protected from the environment. We shall discuss this point further in Chapter 3.
The increased incorporation of [^{14}C]oleic acid into the cells during inhibition of peptidoglycan synthesis is not specific for oleic acid as a lipid precursor. When oleic acid was replaced by palmitic acid, the results were similar but less pronounced. This can be explained by considering the relative amount of free palmitic acid in the normal and inhibited cells. The amount of label found in the pool of free palmitic acid in normal cells, compared to the inhibited cells, was relatively much higher. During inhibition of peptidoglycan synthesis much of this free fatty acid pool is consumed by an increased incorporation into the other lipid fractions. The high concentrations of free palmitic acid in normal cells caused that the increase of incorporation of palmitic acid into lipids during inhibition of cell wall biosynthesis was less noticeable than for oleic acid. We cannot conclude from these experiments that during the increased incorporation of fatty acids the net lipid synthesis also increased.
During inhibition of peptidoglycan biosynthesis the rate of [^{3}H]glycine incorporation into cellular protein and into the membrane fraction was relatively low, compared with normal cells. This was especially apparent in cells inhibited with penicillin G, vancomycin and bacitracin. This may be due to secondary effects like the occurrence of cell damage because of inhibition of cell wall synthesis or changes in the permeability of the membrane which alter rates of uptake for extracellular amino acids may be the cause. The latter possibility can be due to a direct action of the antibiotics on the membrane or to a general effect of the inhibition of cell wall synthesis on the membrane.
No morphological indications for the occurrence of extensive cell damage during inhibition of cell wall synthesis were observed. Observations by phase-contrast microscopy and by electron microscopy of fixed and stained thin sections (5.3) also revealed no cellular damage such as lysis of the cells. If extensive cell damage would have occurred it would have to be a general effect on the synthesis

of not only proteins but also of other components such as lipids. Lipid synthesis continued also during inhibition. These considerations suggest that the observed inhibition of protein synthesis may be correlated with a diminished rate of uptake or a lowered accumulation capacity for precursors. It is also suggestive to note that inhibition of peptidoglycan synthesis with several structurally different antibiotics with different modes of action, as well inhibition by omission of growth factor, all resulted in inhibition of protein synthesis. There was only a difference in the extent of inhibition. It is interesting to mention the results of Holden (1965), who found that penicillin inhibited the accumulation capacity of amino acids in *L. plantarum* and that a reduced capacity for amino acid accumulation by vitamine B_6-deficient cells arises from a failure to synthesize a normal cell wall. This suggestion agrees with the observations we made, if we suppose that the inhibition of protein synthesis originates from a reduced accumulation capacity for amino acids.

We find no clear effects of sodium acetate omission on the cells. Remarkable is the relatively large glucosamine-labeled fraction in the cells, which are resistant to lysozyme-digestion and the lysozyme-resistant cells, after cultivation in medium depleted from sodium acetate. This may be due to a structural change in the cellular peptidoglycan which alters its susceptibility for lysozyme. Such a change can be expected theoretically since the cells are exposed to a medium of relatively low osmotic pressure. A more rigid form of the peptidoglycan may be needed for cellular survival under these conditions. An other possibility might be that by lack of acetate the peptidoglycan contains less N-acetylated residues. Araki et al. (1971) showed for strains of *B. cereus* with cell walls, which were completely insensitive to lysozyme, that the peptidoglycan contained large amounts of non-N-substituted glucosamine residues. The lysozyme-resistant cell walls were converted to a sensitive form by acetylation with acetic anhydride. This indicated that the resistance of the cell walls to lysozyme is ascribable to the occurrence of non-N-substituted glucosamine residues in the peptidoglycan.

The lipid synthesis continued at a considerable rate during depletion of sodium acetate. The lipid synthesis in the membrane fraction also

continued, despite the inhibition of protein synthesis, in the late incubation time. This is again in agreement with the results obtained for lipid synthesis in cells in which protein synthesis was inhibited.

Depletion of Mg^{2+} ions from the culture medium resulted in inhibition of protein and lipid synthesis. Though the absorbance of the normal culture showed a higher increase than that of the inhibited culture the incorporation of N-benzoyl-glucosamine was of the same order in both cultures. This implies that a Mg^{2+}-depleted cell may have a larger peptidoglycan production than a normal growing cell.

In this chapter we have studied several synthetic processes in the cell wall and the cell membrane in relation to each other. However, the results are subject to some restrictions. These are:

(i) the synthetic rates found for the different processes do not necessarily reflect a net synthesis. Since turnover processes may also be responsible for the incorporation of precursors, it is important to know the metabolic stability of the different cellular components;

(ii) besides knowing the turnover of the lipids, it is important to know whether the incorporation of fatty acids is influenced by changes in the cellular fatty acid pattern.

CHAPTER 3

THE METABOLIC STABILITY OF CELL WALL AND CELL MEMBRANE COMPONENTS

3.1. INTRODUCTION

In Chapter 2 we have described experiments in which the incorporation of cellular precursors has been studied under conditions of growth inhibition. The aim of the study was to obtain information about the processes of cell wall and cell membrane formation under conditions which disturbed these processes.

The most important results were:

(i) during inhibition of protein synthesis there is still a considerable incorporation of oleic acid into membrane lipids;

(ii) during inhibition of protein synthesis considerable incorporation of glucosamine into cell wall peptidoglycan occurs;

(iii) during inhibition of cell wall peptidoglycan synthesis there is an increased incorporation of oleic acid into lipids.

For an adequate interpretation these results need to be completed with information about the turnover of the cellular components. Apparent incorporation of precursors may be due to net synthesis of cell materials, but it may also be due to turnover of molecules. Changes in incorporation of precursors may be caused by changes in turnover rates.

In this chapter we shall confine ourselves to the metabolic stability of cellular components during normal and inhibited growth. In order to investigate this matter, cells labeled with radioactive precursors were incubated in non-radioactive culture media under normal and growth-inhibitory conditions. The decrease of label during incubation was studied.

If degradation products of cellular components are not completely reutilized a turnover will be observed in our system. If there is turnover of components with complete reutilization of the break-down products it will not be observable. Our experiments do not differentiate between "no turnover" and turnover with complete reutilization of components.

3.2. MATERIALS AND METHODS

Unless otherwise mentioned, the methods and materials are identical to those described in Chapter 2.

3.2.1. Turnover experiments

Cells from 10 h-cultures were incubated with radioactive precursors (per ml culture were added: 15.0 nCi [^{3}H]glycine, 50.0 nCi N-benzoyl-[^{14}C]glucosamine, 4.9 nCi [^{14}C]oleic acid and 3.2 nCi [^{14}C]palmitic acid) during 60 min in fresh culture medium (pH 6.8) (see for incubation procedure *2.2.13*). Cells were collected by centrifugation at 10 000 g during 15 min and washed with 2 vol of culture medium without human milk. The labeled cells were resuspended in fresh non-radioactive culture medium, containing 25 µg/ml N-benzoyl-glucosamine (except in experiments in which N-benzoyl-glucosamine omission was studied) with and without antibiotics added. For experiments in which the omission of Mg^{2+} or sodium acetate was studied the cells were resuspended in medium without Mg^{2+} or sodium acetate, containing 25 µg/ml N-benzoyl-glucosamine. The absorbance (550nm) of the culture was about 1.0 at the start of the experiments. Incubation of the cells was performed at 37^{o} under N_2/CO_2 gas. The incorporated radioactivity was measured in cells isolated from samples of 2.5 ml as described in *2.2.14 - 2.2.16*.

3.2.2. Extraction of lipids from extracellular fluid

Samples of 2.5 ml culture (in some experiments 200 ml) were mixed with an equal volume medium of 0^{o} and centrifuged at 10 000 g for 15 min at 4^{o}. Lipids were extracted from the supernatant according to Bligh and Dyer (1959). One vol supernatant was intensively mixed with 2.8 vol methanol and 1.4 vol chloroform. After 1 h at room temperature, 1 vol chloroform and 1 vol water were added. The organic phase and the aqueous phase were intensively mixed and separated by centrifugation. The aqueous phase was removed and the organic phase was collected. For determination of radioactivity the

solvent was evaporated in a scintillation vial and 10 ml omnifluor-toluene scintillation fluid was added. For chromatographic analysis the solvent was evaporated *in vacuo* and the lipids were redissolved in chloroform-methanol (1:1, by vol).

3.2.3. Extraction of lipids from cells

Cells from 100 ml culture were centrifuged and washed with 1 vol fresh culture medium (pH 6.8) at 4°. Washed cells were resuspended in 1 vol 0.2 M acetate buffer (pH 5.0) and 2.8 vol methanol and 1.4 vol chloroform were added successively. After vigorous mixing the system was allowed to stand at room temperature for 1 h. The extract was separated from the residue. The cell residue was extracted three times with 0.5 vol chloroform-methanol (2:1, by vol). The extracts were combined and separation into two phases was achieved by adding 1 vol chloroform and 1 vol distilled water. After mixing intensively, the two phases were allowed to form a clear separation and the lower (organic) phase was collected. The aqueous phase was washed once with 1 vol chloroform. The combined extracts were evaporated *in vacuo* and dissolved in a small volume of chloroform-methanol (2:1, by vol).

3.2.4. Measurement of radioactivity in the hot TCA-insoluble fraction of cells and extracellular fluid

Cells were labeled with radioactive precursors and incubated as described in *3.2.1*. Samples (2.5 ml) were collected and mixed immediately with 1 vol culture medium without human milk, containing 1% bovine serum albumin as a carrier protein at 0°. Cells were centrifuged for 15 min at 10 000 g at 4°. One vol of supernatant was added to 1 vol 10% TCA of 0°. Radioactivity insoluble in hot TCA was determined described in 2.2. The cells were washed with 2 vol culture medium and resuspended in 1 vol culture medium. One vol 10% TCA was added and the radioactivity insoluble in hot TCA was determined as descrined in *2.2.14*.

3.2.5. *Incorporation of [^{32}P]phosphate*

[^{32}P]phosphate was added to cultures in an amount of 1 mCi/l carrier-free [^{32}P]orthophosphate. The radioactive phosphate was purchased from Philips-Duphar (Petten, The Netherlands). To enhance the incorporation, the concentration of potassium phosphate in the culture medium was decreased ten-fold (from 2.5 to 0.25 g/l).

3.2.6. *Silicic acid column chromatography*

Silicic acid (Mallinkrodt, St. Louis, U.S.A.) was purified in portions of 450 g by washing with 1 l each of petroleum ether (40^{o}-60^{o}), chloroform, methanol and acetone successively and dried at 100^{o} for 16 h. Purified silicic acid was activated for 16 h at 120^{o}. After cooling to room temperature the activated silicic acid was suspended in chloroform and poured into columns. The columns were washed with chloroform before use. Lipids were dissolved in chloroform and applied to the column. The maximal amount of lipid applied to the column was 10 mg per g silicic acid. The lipid material was washed into the silicic acid with small volumes of chloroform. Separation of the lipids was achieved by eluting the column with successively: chloroform, acetone, methanol and chloroform-methanol-water (10:10:1, by vol). The chloroform fraction contained the neutral lipids, the acetone fraction the glycolipids and the last two fractions contained the phospholipids.

3.2.7. *Thin-layer silica gel chromatography*

a. ^{32}P-labeled lipids

^{32}P-labeled lipids were analyzed by two-dimensional chromatography on thin-layer silica gel G plates. The chromatograms were developed with chloroform-methanol-7 M ammonia (60:35:5, by vol) in the first direction and with chloroform-methanol-acetic acid-water (125:37:9.5:1.5, by vol) in the second direction. Radioactive spots were located by autoradiography using Kodak No Screen X-ray film and scraped into scintillation vials. Radioactivity was determined by

scintillation counting after mixing with 5 ml water and 10 ml Instagel.

b. [^{14}C]glycolipids

[^{14}C]glycolipids were analyzed by thin-layer silica gel chromatography with chloroform-methanol-7 M ammonia (70:20:2, by vol) as the solvent. As reference compounds were used [^{14}C]galactosyldiglycerides and unlabeled glycolipid components. Radioactive spots were detected by autoradiography, scraped off the plates and transferred to scintillation vials. After mixing with 5 ml water and 10 ml Instagel, radioactivity was determined by liquid scintillation counting. Unlabeled reference glycolipids were detected on the plates with the periodate-Schiff reagent (Baddiley et al., 1956).

3.3. RESULTS

3.3.1. Turnover of components during normal growth

The turnover of glycine in cellular protein, of glucosamine in cell wall peptidoglycan and of oleic acid in the cellular lipids was studied. Cells were labeled during 60 min with the radioactive precursors: [^{3}H]glycine, N-benzoyl-[^{14}C]glucosamine and [^{14}C]oleic acid. Non-incorporated precursors were removed by washing the labeled cells with non-radioactive culture medium. The washed cells were resuspended in fresh culture medium and growth was allowed to continue. No significant retardation of growth was observed. Fig. 35 shows the results of a representative turnover study during normal growth. The increase of absorbance in the culture during a 90 min incubation period was about 85%, which is near the 100% increase often found during a 90 min incubation of exponential growing cells in fresh culture medium at 37°. During growth of the bacteria no significant decrease of [^{14}C]glucosamine label in the peptidoglycan fraction was observed. In the beginning of the incubation period a small increase in radioactivity in the cells was found. This is probably due to the incorporation of intracellular free precursors into the peptidoglycan.
The amount of |^{3}H|glycine label in the cellular protein and [^{14}C]oleic

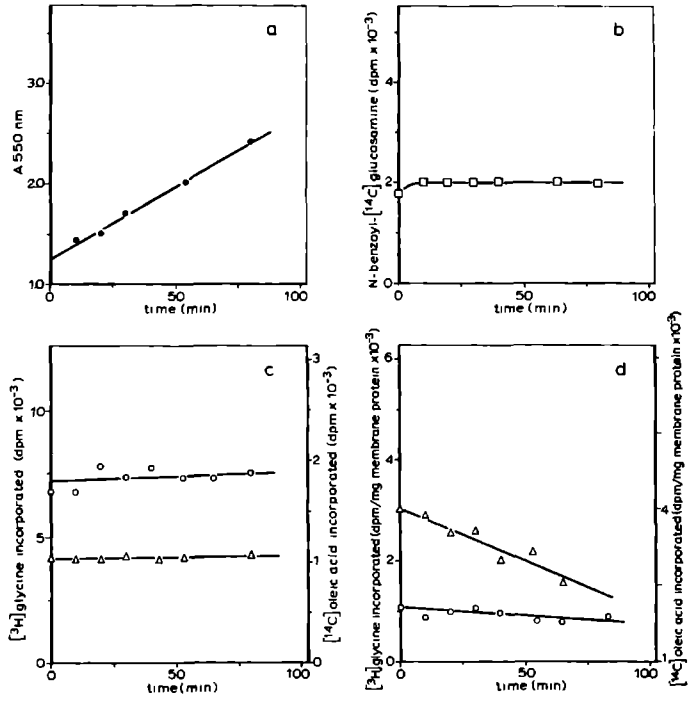

Fig. 35. Turnover of components during normal growth. Cells of a 10 h culture were labeled with [^{3}H]glycine, N-benzoyl-[^{14}C]glucosamine and [^{14}C]oleic acid and resuspended in fresh non-radioactive culture medium (pH 6.8). Cells were incubated at 37° under N_2/CO_2 and the radioactivity remaining in the cells was determined as described in *3.2.1.*

a. Absorbance of the culture;
b. N-benzoyl-[^{14}C]glucosamine incorporated;
c. [^{3}H]glycine incorporated (Δ-Δ); [^{14}C]oleic acid incorporated (o-o);
d. [^{3}H]glycine (Δ-Δ) and [^{14}C]oleic acid (o-o) incorporated in the membrane fraction per mg membrane protein.

acid label in the cellular lipids was also preserved during growth. There was no detectable decrease of label in these cells.
The ratio of the [^{3}H]glycine incorporated in the membrane to the membrane protein mass falls during 90 min of growth to about 54% of the value at the start of the incubation. Considering the nature of the parameter used for the membrane data, an isotope "dilution" effect

caused by an increase of the amount of membrane protein must be involved. If there is no turnover of glycine in the membrane protein and if one assumes that an 85% increase of the bacterial density implies also an 85% increase in the total amount of membrane and membrane protein, a decrease to 58% of the original value should be expected. The absence of turnover of glycine in the total cellular protein and the agreement of the calculated and observed values for isotope "dilution" in the membrane suggest a lack of turnover of glycine in the membrane protein.

The amount of [^{14}C]oleic acid per mg membrane protein was rather constant during growth. Since a membrane dilution effect can also be expected in this case, it is remarkable that a significant decrease of [^{14}C]oleic acid per mg membrane protein was not found. The amount of oleic acid label in the total cell remained constant during incubation. The absence of an apparent membrane "dilution" effect implies a supply of label from extra-membranous sources to the membrane. Potential sources could be the cytoplasmic lipids in the cells, since we have found that a significant fraction of the label is in the neutral lipids.

In these studies of normal growth we have observed the absence of turnover for cellular protein, peptidoglycan and cellular lipids. This does not imply the complete absence of turnover. The possibility exists that turnover is taking place, but the components are completely reutilized, or that the time of incubation or labeling was too short. The results obtained in the study of the turnover of the various cellular components will be used for the interpretation of the incorporation data for precursors, described in Chapter 2. In all subsequent turnover studies only turnover data obtained for normal cells and inhibited cells will be compared.

3.3.2. Turnover of protein and peptidoglycan

Cells, labeled with [^{3}H]glycine and N-benzoyl-[^{14}C]glucosamine were further incubated in non-radioactive culture medium in the presence or absence of growth inhibitors, or in media from which components had been omitted. During incubation in the non-radioactive medium

the amount of radioactivity still incorporated into the hot TCA-insoluble fraction was determined.
We could not find a significant decrease of glucosamine label during normal growth, during inhibition of protein and peptidoglycan biosynthesis and during omission of Mg^{2+} and sodium acetate from the culture medium (Fig. 36). There was also no significant decrease of [^{3}H]glycine label under the experimental conditions mentioned.

3.3.3. *Turnover of oleic acid-containing lipids in cells during growth inhibition*

Cells were labeled with [^{14}C]oleic acid and further incubated in non-radioactive culture medium in the presence or absence of growth inhibitors, or in media from which nutrients had been omitted. The amount of radioactivity still present in the cells during incubation was determined (Fig. 37). During normal growth of the cells the amount of [^{14}C]oleic acid label was conserved in the cells. There was no significant decrease of radioactivity during incubation. The same observation is valid for incubation of the cells in the presence of chloramphenicol and tetracyclin. However, we found a significant decrease of radioactivity in the cells during incubation with all antibiotics or conditions which caused an inhibition of cell wall peptidoglycan biosynthesis. Actinomycin-D action caused a decrease of 30% of the initial radioactivity during 90 min incubation. As described in Chapter 2, the inhibitory action of actinomycin-D is primarily on the protein biosynthesis, but in our system there was also inhibition of the peptidoglycan biosynthesis.
Penicillin G caused a loss of 76% of the initial radioactivity during 90 min of incubation. These figures were for bacitracin: 61%, vancomycin: 75%, D-cycloserine: 40%, N-benzoyl-glucosamine omission from the culture medium: 36%, and staphylococcin 1580: 66%.
The conclusion is that there is no significant turnover of oleic acid-containing lipids during normal growth, during inhibition of protein synthesis with chloramphenicol and tetracyclin and during omission of Mg^{2+} and sodium acetate from the culture medium. However, during inhibition of peptidoglycan synthesis a significant turnover of

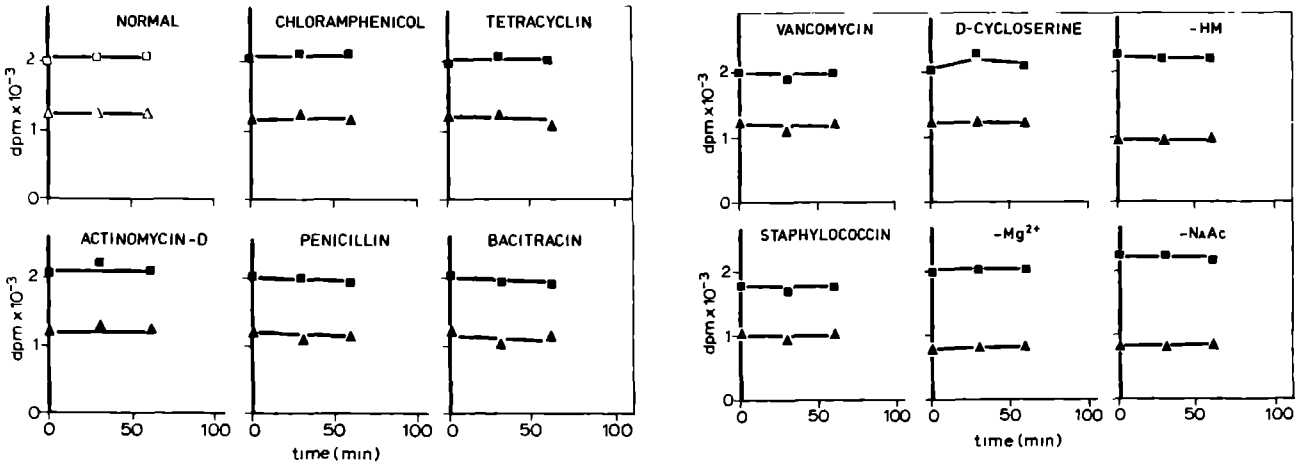

Fig. 36. Turnover of [^{3}H]glycine-labeled protein and [^{14}C]glucosamine-labeled peptidoglycan. Cells were labeled with [^{3}H]glycine and N-benzoyl-[^{14}C]glucosamine and resuspended in fresh, non-radioactive culture medium (pH 6.8). Cells were incubated at 37° under N_2/CO_2 gas under normal conditions and in the presence of antibiotics. Cells were also incubated in media from which sodium acetate, Mg^{2+} or N-benzoyl-glucosamine (-HM) were omitted. The radioactivity present in the hot TCA-insoluble fraction of the total culture was determined. See for experimental details *3.2.1.*

(□-□) N-benzoyl-[^{14}C]glucosamine incorporated; (Δ-Δ) [^{3}H]glycine incorporated.

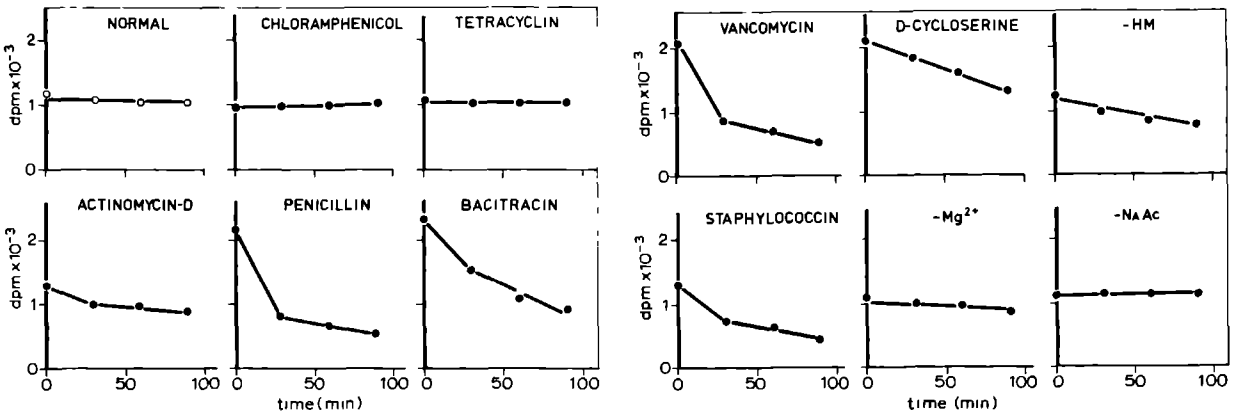

Fig. 37. Turnover of oleic acid-containing lipids during inhibition of growth. Cells were labeled with [^{14}C]oleic acid and resuspended in fresh, non-radioactive culture medium (pH 6.8). Cells were reincubated at 37° under N_2/CO_2 gas under normal conditions and in the presence of antibiotics. Cells were also incubated in media from which sodium acetate, Mg^{2+} or N-benzoyl-glucosamine (-HM) were omitted. The radioactivity in the cells was determined. See for experimental details *3.2.1.*

oleic acid takes place. This turnover is observed with inhibitors of peptidoglycan synthesis at different levels and also when inhibition of peptidoglycan synthesis is realized by depletion of N-benzoyl-glucosamine.

3.3.4. Turnover of palmitic acid-containing lipids in cells during inhibition of cell wall peptidoglycan biosynthesis

Our preceding experiments indicated an increased turnover of oleic acid-containing lipids under conditions of inhibited peptidoglycan biosynthesis. We wanted to know whether the increase in turnover could be found with other fatty acids. The turnover experiments were therefore repeated with [^{14}C]palmitic acid (Fig. 38).
The ^{14}C-activity in the cells remained at the same level during incubation under normal conditions, hence there was no apparent turnover, like found for oleic acid-labeled cells. This means that under normal conditions there is either no significant turnover or there might be turnover of the fatty acid with complete reutilization. When the biosynthesis of cell wall peptidoglycan was inhibited, a drastic decrease of palmitic acid label in the cells during incubation could be observed. Penicillin G decreased the initial radioactivity present in the cells during an incubation period of 90 min by about 65%, vancomycin by 50%, bacitracin by 38%, D-cycloserine by 29% and omission of N-benzoyl-glucosamine by 37%. These results are identical with those demonstrated for [^{14}C]oleic acid-labeled cells under conditions of inhibition of peptidoglycan biosynthesis. The effect of an increased turnover compared to normal cells is thus not limited to oleic acid but occurs also for palmitic acid. So at least the two quantitatively most important fatty acids of the lipids of the organism are involved in this process.

3.3.5. Turnover of glycine and oleic acid in the cytoplasmic membrane

The amount of [^{3}H]glycine and [^{14}C]oleic acid label in the cytoplasmic membrane fraction was studied after incubation of labeled cells in non-radioactive culture medium, in the presence and absence of growth inhibitors, or under growth-inhibitory conditions. We have described

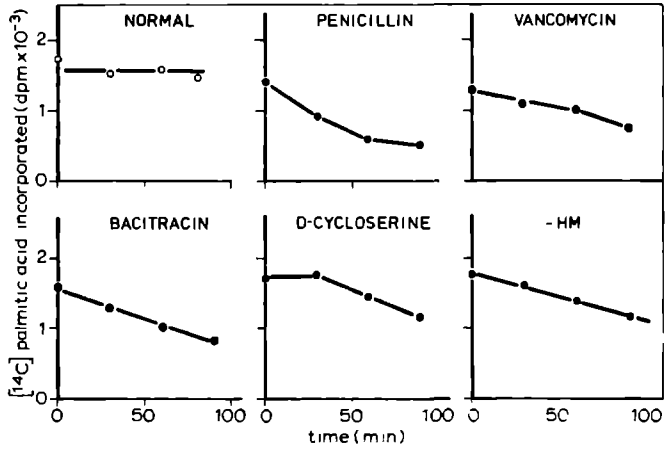

Fig. 38. Turnover of palmitic acid-containing lipids during inhibition of peptidoglycan biosynthesis. Cells from 10 h-cultures were labeled with [^{14}C]palmitic acid and resuspended in fresh, non-radioactive culture medium (pH 6.8). Cells were incubated at 37° under N_2/CO_2 gas without inhibitors and in the presence of antibiotics. Cells were also incubated in culture media from which N-benzoyl-glucosamine was omitted (-HM). The radioactivity present in the cells was determined. See for experimental details *3.2.1.*

the observation for normal cells in *3.3.1.* In all the experiments described in this section, normal cells were tested for comparison. There was no decrease of [^{3}H]glycine per mg membrane protein during incubation with chloramphenicol, tetracyclin and actinomycin-D (Fig. 39). In the normal cells the decrease of label per mg membrane protein was as could be expected on the basis of increase of membrane protein ("isotope dilution"). These results suggest that there is no significant turnover of glycine in the membrane and no increase

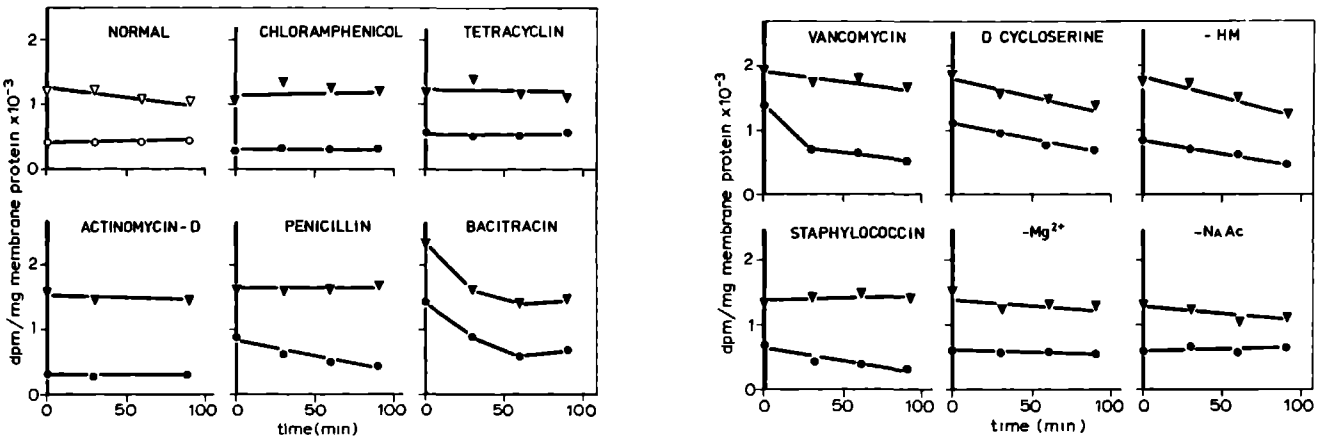

Fig. 39. Turnover of protein and lipids in the membrane fraction. Cells from 10 h-cultures were labeled with [^{3}H]glycine and [^{14}C]oleic acid and resuspended in fresh, non-radioactive culture medium (pH 6.8). Cells were incubated at 37° under N_2/CO_2 gas under normal conditions and in the presence of antibiotics. Cells were also incubated in media from which sodium acetate, Mg^{2+} and N-benzoyl-glucosamine (-HM) were omitted. The radioactivity present in the membrane fraction per mg membrane protein was determined. See for experimental details *3.2.1.*

(▽-▽) [^{3}H]glycine in membrane fraction per mg membrane protein; (o-o) [^{14}C]oleic acid in membrane fraction per mg membrane protein.

in membrane protein during inhibition of protein synthesis, or that the decrease of [^{3}H]glycine label is proportional with a decrease in membrane protein mass.

Penicillin G inhibition showed the same results for glycine as the inhibitors of protein synthesis. There is no significant turnover of glycine in the membrane fraction during inhibition with penicillin G. Since we know from the experiments described in Chapter 2 that during inhibition of cells with penicillin G glycine incorporation is also severely inhibited, we cannot expect an important increase of the membrane protein mass in the cells during incubation.
Bacitracin inhibition decreased the amount of [^{3}H]glycine per mg membrane protein by 37% during 90 min incubation. The decrease is much more than in the normal cells. Theoretically we could explain the observation by assuming that there is an increased membrane protein formation in respect to the label present, or by assuming an increased turnover of glycine. It is reasonable to expect that membrane protein formation will not exceed that in the normal cells, since we found that glycine incorporation is strongly inhibited (Chapter 2). The results are indicative for an increased turnover of glycine in the membrane.

During inhibition with vancomycin and D-cycloserine and by omission of N-benzoyl-glucosamine from the culture medium, there was a decrease of the amount of [^{3}H]glycine per mg membrane protein which was of the same order of that for normal cells. Together with the results of the [^{3}H]glycine incorporation experiments of Chapter 2 it can be concluded that the increase of membrane protein is lower than in normal cells. The decrease in [^{3}H]glycine per mg membrane protein is partly due to net synthesis and partly to an increased turnover of glycine in the membrane proteins.

Staphylococcin 1580 action showed the same result as the inhibitors of protein biosynthesis. As we know from the experiments of Chapter 2 the glycine incorporation is severely affected by staphylococcin 1580 action. We may therefore expect that the membrane protein mass will not significantly increase suggesting that there is no significant

turnover of glycine in the membrane protein during staphylococcin action.

During omission of Mg^{2+} and sodium acetate from the culture medium the amount of [^{3}H]glycine per mg membrane protein decreased. The decrease was smaller than found in normal cells and did not suggest an increased turnover of glycine in the membrane protein compared to normal cells.

During inhibition of protein biosynthesis with chloramphenicol, tetracyclin and actinomycin-D the amount of [^{14}C]oleic acid per mg membrane protein remained constant (Fig. 39). These results are identical with those obtained during normal growth. During inhibition of peptidoglycan synthesis a significant decrease of [^{14}C]oleic acid per mg membrane protein was observed. The following decreases were found, expressed as the percentage of the initial value after 90 min incubation: 55% with penicillin G, 50% with bacitracin, 63% with vancomycin, 39% with D-cycloserine, 45% after omission of N-benzoylglucosamine and 50% with staphylococcin 1580.

The large decrease of the [^{14}C]oleic acid content of the membrane fraction compared to the constant value during normal growth can be due to the following points:

(i) the [^{14}C]oleic acid label is more diluted in the inhibited cells through a larger increase of the membrane protein mass;

(ii) there is an increased turnover of [^{14}C]oleic acid in the membrane.

From the experiments, described in Chapter 2, we know that during inhibition of peptidoglycan synthesis under our experimental conditions glycine incorporation into cellular and membrane protein is inhibited, compared to normal cells. Taken together with the observed turnover, we cannot expect a larger increase of membrane protein in inhibited cells than in normal cells. The second possibility is more probable. This would also agree with the observed turnover of [^{14}C]oleic acid in the total cellular lipids during inhibition of peptidoglycan synthesis.

An exception is formed by actinomycin-D inhibited cells, In the total cellular lipids there was a small turnover observable during inhibition with actinomycin-D, whereas the amount of [^{14}C]oleic acid per

mg membrane protein remained constant. It might be possible that the turnover effect is too small for an accurate detection in the membrane fraction. Another possibility might be that the decrease of [^{14}C]oleic acid label in the membrane is proportional to a decrease in the membrane protein mass.
During omission of Mg^{2+} and sodium acetate from the culture medium the [^{14}C]oleic acid per mg membrane protein remained constant.
We shall discuss the consequences of these results in the last section of this chapter.

The turnover experiments are difficult to interpret since the values are expressed on the basis of the membrane protein content. However, the results suggest the following:

(i) there is no significant turnover of membrane protein during normal growth;

(ii) there is no significant turnover of membrane protein during inhibition of protein biosynthesis;

(iii) there is no increased turnover of oleic acid-labeled membrane lipids during inhibition of protein synthesis;

(iiii) there is a considerable turnover of membrane protein during inhibition of peptidoglycan biosynthesis by bacitracin and possibly also a small turnover during inhibition of peptidoglycan synthesis by vancomycin;

(iiiii) there is a markedly increased turnover of oleic acid-labeled membrane lipids during inhibition of peptidoglycan synthesis;

(iiiiii) there is no increased turnover of membrane oleic acid and membrane protein during omission of Mg^{2+} and sodium acetate from the culture medium.

3.3.6. Appearance of lipids in the culture medium during inhibition of peptidoglycan biosynthesis

The turnover experiments with [^{14}C]oleic acid and [^{14}C]palmitic acid labeled cells indicated a loss of radioactivity during inhibition of cell wall peptidoglycan synthesis. Since all original radioactivity is incorporated in lipid-soluble material, we may assume that during inhibi-

tion lipids or their derivatives are lost from the cell. Theoretically lipids can be lost from the cell in the form of intact lipids or after conversion of lipids into water-soluble products which are released into the culture medium. The former possibility includes excretion of fatty acids as well excretion of more complex lipid components.

We checked the possibility that lipids might be excreted to the medium. After inhibition of peptidoglycan synthesis of cells, prelabeled with [^{14}C]oleic acid, a significant amount of label was found in lipids isolated from the medium. This indicates that lipids were excreted from the cells into the culture medium. The process of lipid excretion was studied by measuring the disappearance of radioactivity from oleic acid-labeled cells during inhibition and the appearance of radioactivity in the lipids extracted from the culture medium.

Fig. 40 shows the results found for normal growth and inhibition of peptidoglycan synthesis. We did not find a significant decrease of radioactivity from cells labeled with [^{14}C]oleic acid during 90 min of incubation at normal growth. Neither did we find a significant amount of radioactivity in the lipids extracted from the medium. However, when cells were inhibited by penicillin G, bacitracin, D-cycloserine, vancomycin, by staphylococcin 1580 or by depletion of N-benzoyl-glucosamine, we found a large decrease in radioactivity in the cells and a concomitant increase of radioactivity in the medium lipids. The greater part of the radioactivity lost from the cells was found in the medium lipids, but in most cases the loss of radioactivity exceeded the amount of radioactivity recovered in the medium lipids. So the possibility exists that part of the cellular lipids is lost from the cell by conversion to water-soluble products. Another possibility might be that excreted lipids adhere to the cell surface before release into the medium. These lipids might remain adhering to the cells after separation from the culture medium, but they could be removed from the cells during the washing process. We did not check these possibilities. Our conclusion from these experiments is that under conditions of inhibited cell wall peptidoglycan synthesis significant amounts of the cellular lipids are lost to the culture medium, and that the possibility is

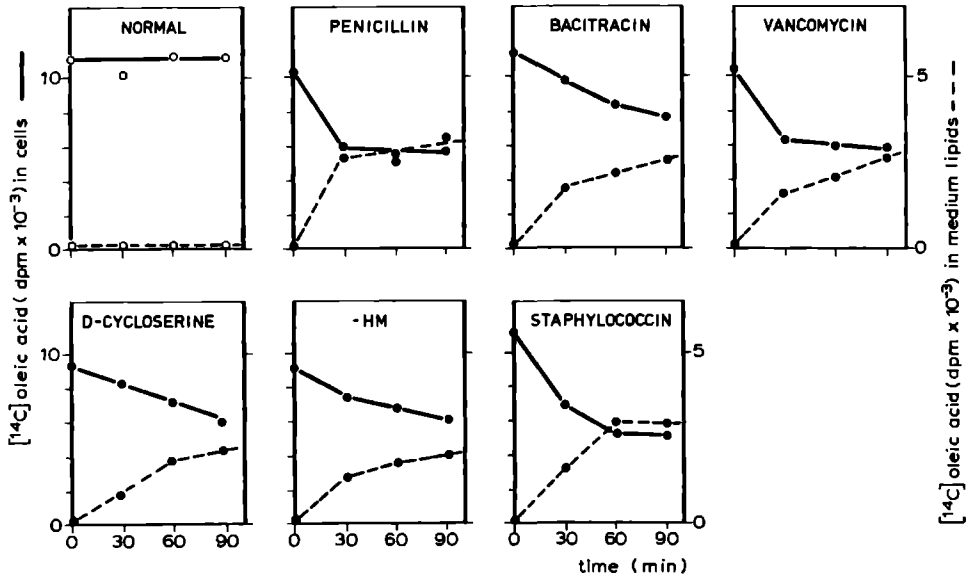

Fig. 40. Appearance of lipids in the culture medium during inhibition of peptidoglycan biosynthesis. Cells from 10 h-cultures were labeled with [^{14}C]oleic acid and resuspended in fresh, non-radioactive culture medium (pH 6.8). Cells were incubated at 37° under N_2/CO_2 gas without growth inhibitors and in the presence of antibiotics. Cells were also incubated in medium from which N-benzoyl-glucosamine was omitted (-HM). Cells and culture fluid were separated. Radioactivity in cells and lipids of culture fluid was determined. See for experimental details *3.2.1* and *3.2.2*.

is not excluded that disappearance of lipids may also occur by other ways.

We investigated whether the appearance of radioactivity in the lipids of the medium also occurred, when [^{14}C]palmitic acid-labeled cells are incubated under conditions of inhibited cell wall biosynthesis (Fig. 41). The results are in line with those found for [^{14}C]oleic

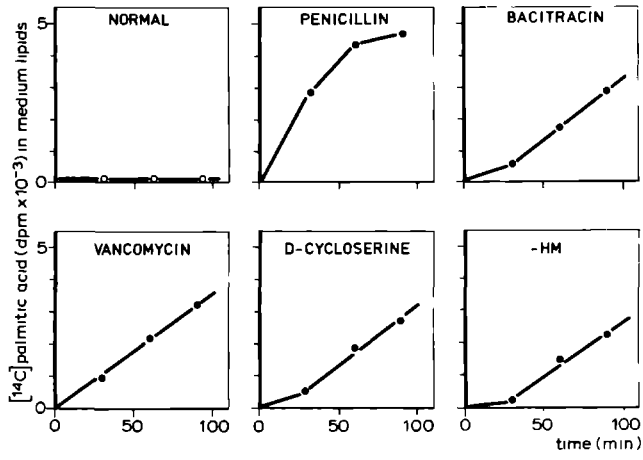

Fig. 41. Appearance of lipids in the culture medium during inhibition of peptidoglycan biosynthesis. Cells from 10 h-cultures were labeled with [^{14}C]palmitic acid and resuspended in fresh, non-radioactive culture medium (pH 6.8). Cells were incubated at 37^{o} under N_2/CO_2 gas without growth inhibitors and in the presence of antibiotics. Cells were also incubated in a medium from which N-benzoyl-glucosamine was omitted (-HM). Cells and culture fluid were separated and the radioactivity in the lipids of the culture fluid was determined. See for experimental details *3.2.1* and *3.2.2*.

acid-labeled cells. During incubation of labeled cells with penicillin G, bacitracin, D-cycloserine, vancomycin and during omission of N-benzoyl-glucosamine from the culture medium we found a progressive labeling of the lipids extracted from the culture medium, whereas under normal conditions there is no significant labeling of the medium lipids during a 90 min incubation.

3.3.7. Loss of protein and peptidoglycan from the cells during growth inhibition

During inhibition of cell wall peptidoglycan biosynthesis there was an extensive loss of lipids to the medium. We were interested in the possibility that along with the loss of lipids other cellular components were also lost to the medium. Such a loss could occur upon damage or lysis of the cells with subsequent release of cellular components.

In *3.3.2* we described the results of turnover experiments for the protein and peptidoglycan of the culture during growth inhibition. We found no significant turnover of these components during normal growth and growth inhibition. However, in those experiments we determined the turnover in cells and culture medium together. The experiments did not give information about the loss of protein and peptidoglycan from the cells to the medium. To check this we labeled cells with [^{3}H]glycine and with N-benzoyl-[^{14}C]glucosamine for 1 h and incubated the labeled cells in non-radioactive medium in the presence of growth inhibitors or in medium from which certain components had been omitted. Cells were separated from the culture medium and were washed. Radioactivity in the hot TCA-insoluble fraction was determined both in the culture medium and the washed cells (Fig. 42). The loss of radioactivity from the cells and the increase of label in the culture medium may give information about loss of protein and peptidoglycan to the medium. During normal growth there was no significant decrease in radioactivity in the hot TCA-insoluble fraction of the cells and also there was no significant appearance of radioactivity in the hot TCA-insoluble fraction of the culture medium. The same results were obtained when cells were incubated in the presence of chloramphenicol, tetracyclin, penicillin G, vancomycin, during depletion of N-benzoyl-glucosamine and in the presence of staphylococcin 1580. During incubation of cells with actinomycin-D and bacitracin there was a slight decrease in glycine label. The decrease was about 10% after 90 min of incubation. There was no radioactivity found in the fraction of the medium insoluble in hot TCA. However, it is possible that some proteins are lost from the

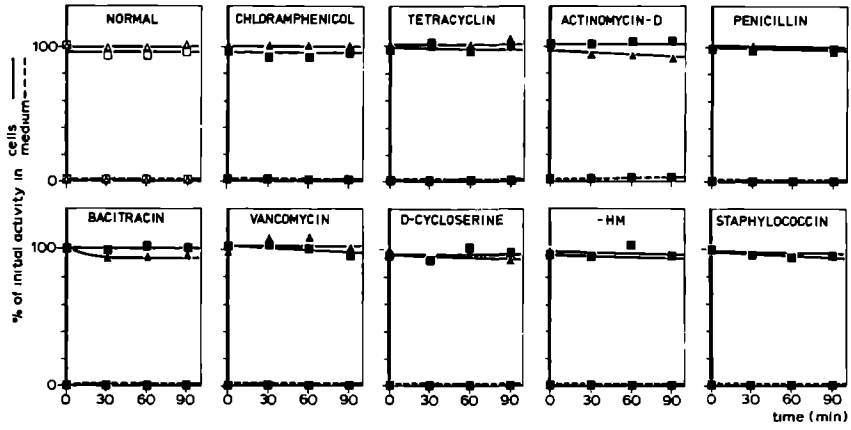

Fig. 42. Loss of protein and peptidoglycan from the cells during inhibition of growth. Cells of 10 h-cultures were labeled with [^{3}H]glycine and N-benzoyl-[^{14}C]glucosamine and resuspended in fresh, non-radioactive culture medium (pH 6.8). Cells were incubated at 37° under N_2/CO_2 gas without inhibitors of growth and in the presence of antibiotics. Cells were also incubated in culture medium from which N-benzoyl-glucosamine was omitted (-HM). Cells and culture medium were separated and incorporation of radioactivity into the hot TCA-insoluble fraction of cells and medium was determined (3.2). Radioactivity is expressed as the percentage of radioactivity originally present in the cells.
[^{3}H]glycine incorporated (Δ-Δ); N-benzoyl-[^{14}C]glucosamine incorporated (□-□).

cells, adsorbe to the cells and thus are not found in the extracellular fluid after separation of the cells from the culture medium. During subsequent washing of the cells before TCA-precipitation the proteins may be removed. Also turnover of protein may give soluble products. These experiments show that during inhibition of protein and cell wall biosynthesis, there are generally no extensive losses of protein and peptidoglycan from the cells to the medium. These results are in contrast with the observation that during inhibition of cell

wall peptidoglycan biosynthesis drastic losses of cellular lipids to the culture medium occur.

3.3.8. Nature of the lipids excreted into the culture medium during inhibition of peptidoglycan synthesis

It was shown in *3.3.6* that during inhibition of peptidoglycan biosynthesis lipids were excreted from the cells into the culture medium. In order to analyze the excreted lipids, the lipids in the cell were labeled with [^{14}C]oleic acid and by $^{32}P_i$. The cells were then cultured under inhibitory conditions for peptidoglycan biosynthesis. The labeled lipids excreted into the medium were isolated and studied.

a. Lipids labeled with [^{14}C]oleic acid

The action of penicillin on cells, prelabeled with [^{14}C]oleic acid during growth for 10 h, was studied after 3 h incubation. The composition of the labeled lipids excreted from the cells and of the cellular lipids was analyzed. We also included an experiment in which the action of penicillin G was studied in culture medium without Tween 80, since this substance contaminates the lipids present in the culture medium and may cause artifacts in the separation of the various lipid components.

About 64% of the radioactivity originally present in the cells is found in the medium lipids after 3 h incubation in the presence of penicillin G. In the absence of Tween 80 this was 43% and during normal growth only 6% (Table VIII). The lower amount of radioactivity in the medium in the absence of Tween 80 may be due to a decreased transfer of cellular lipids to the culture medium. Tween 80 easily solubilizes lipids in a hydrophilic environment and may play a role in the transfer of the lipids from the cells to the culture medium. In the absence of Tween 80 the lipid excretion may be retarded somewhat, or part of the lipids may remain adsorbed to the cellular surface. The marked amount of radioactivity in the medium lipids in the absence of Tween 80 shows that Tween 80 is not essential for the excretion process.

TABLE VIII. [^{14}C]OLEIC ACID LABELED LIPIDS IN CELLS AND CULTURE MEDIUM AFTER INCUBATION WITH PENICILLIN

Cells were cultivated for 10.5 h in the presence of [^{14}C]oleic acid. After washing the cells with non-radioactive culture medium the cells were suspended in fresh, non-radioactive culture medium (pH 6.8), with or without Tween 80 and containing human milk. Penicillin was added in a concentration of 10 µg/ml. The control culture received no penicillin. The cultures were incubated for 3 h at 37° in a N_2/CO_2 atmosphere. After centrifugation of the culture lipids were extracted from the supernatant according to the method of Bligh and Dyer (1959) and from the cells as described in *3.2.8*. Radioactivity in the lipids was determined by liquid scintillation counting and expressed in dpm x 10^{-6}.

Culture	A_{550}		pH		Lipid-^{14}C-activity		
	0 h	3 h	0 h	3 h	medium	cells	total
With penicillin	2.30	2.40	6.80	5.40	7.87	4.37	12.24
With penicillin (-Tween 80)	2.30	2.40	6.80	5.40	5.11	6.80	11.91
No penicillin	2.30	3.97	6.80	5.19	0.65	10.69	11.34

The lipids extracted from the culture medium and the cells were fractionated (Table IX). Only a small part of the excreted lipids from the inhibited cells consisted of neutral lipids. Little free oleic acid was detected in the neutral lipids. The radioactivity of the lipids was mainly found in the phospholipids and the glycolipids. There are no important differences in the distribution of radioactivity over the various lipid fractions due to the influence of Tween 80.

The composition of the labeled lipids in the medium of the control culture was very different from the medium lipids in the inhibited cultures.

TABLE IX. FRACTIONATION OF [^{14}C]OLEIC ACID-LABELED LIPIDS

Labeled lipids from cells and medium were fractionated by silica gel column chromatography (*3.2.6*) into neutral lipids, glycolipids and phospholipids. The radioactivity in each fraction was determined by liquid scintillation counting and expressed as the percentage of the total ^{14}C-radioactivity recovered from each column. Recovery was 75-85%.

	Condition	Neutral lipids	Glyco-lipids	Phospho-lipids
Medium	Penicillin	1.9	32.6	65.4
	Penicillin, -Tween 80	2.6	37.0	60.4
	No penicillin	38.7	24.1	37.2
Cells	Penicillin	12.9	32.5	54.4
	Penicillin, -Tween 80	11.7	30.3	57.9
	No penicillin	8.2	30.4	61.3

In the control culture the largest part of the medium lipids consisted of neutral lipids and phospholipids. The distribution of radioactivity over the various lipid fractions of inhibited cells did not differ much from that for the control cells.

The composition of the medium glycolipids, excreted in the presence of penicillin, shows that all components commonly present in the lipids of *B. bifidum* var. *pennsylvanicus* were present (Table X). There were only small differences in lipid composition due to the presence of Tween 80 in the medium. The labeled glycolipid fraction consists mainly of digalactosyldiglyceride, acyldigalactosyldiglyceride and monogalactosyldiglyceride, while diacylmonogalactosyldiglyceride and trigalactosyldiglyceride are only minor components.

The main differences between the inhibited culture and the control culture are a higher digalactosyldiglyceride content and a lower

TABLE X. COMPOSITION OF [^{14}C]OLEIC ACID GLYCOLIPID FRACTION

[^{14}C]oleic acid-labeled glycolipids were analyzed by thin-layer silica gel chromatography (*3.2.7*). The radioactivity of each component was expressed as percentage of the radioactivity present in all glycolipid components.

Abbreviations: MGD': diacylgalactosyldiglyceride; MGD: monogalactosyldiglyceride; acyl-DGD: acyldigalactosyldiglyceride; DGD: digalactosyldiglyceride; TGD: trigalactosyldiglyceride.

	Condition	Distribution in %				
		MGD'	MGD	acyl-DGD	DGD	TGD
Medium	Penicillin	2.2	28.0	26.5	38.7	4.6
	Penicillin, -Tween 80	1.9	34.4	23.0	35.8	2.7
	No penicillin	4.2	18.9	26.9	45.0	5.1
Cells	Penicillin	2.3	12.9	23.6	48.6	12.6
	Penicillin, -Tween 80	1.9	23.2	21.7	43.0	6.5
	No penicillin	2.7	20.1	30.1	42.2	3.7

monogalactosyldiglyceride content in the control culture medium. While the composition of the glycolipids in medium and cells of the control culture was about the same, significant differences were found between the composition of the labeled lipids in the cells and in the medium of the inhibited cultures. The trigalactosyldiglyceride and digalactosyldiglyceride fractions were higher in the medium than in the cellular lipids, whereas the monogalactosyldiglyceride fraction in the medium was lower than in the cells.

In the phospholipid fraction all phospholipids were found which are common for *B. bifidum* var. *pennsylvanicus* (Table XI).

TABLE XI. COMPOSITION OF [^{14}C]OLEIC ACID PHOSPHOLIPIDS

[^{14}C]oleic acid-labeled phospholipids were analyzed by thin-layer silica gel chromatography. Before application to the plates a part of the lipids was mixed with ^{32}P-labeled lipids. Another portion was applied immediately. Phospholipid location on the plates was determined by autoradiography. After ^{32}P-decay the radioactive spots were assayed for ^{14}C-radioactivity. ^{14}C-activity of the oleic acid-labeled phospholipid fraction appeared to be present only in ^{32}P-containing spots (*3.2.7*). The radioactivity of each component was expressed as percentage of the radioactivity present in all phospholipid components. Analysis was made in triplicate.

Abbreviations: DPG: diphosphatidylglycerol; lyso-DPG: lyso-derivatives of diphosphatidylglycerol; PG: phosphatidylglycerol; GPGD: glycerophosphorylgalactosyldiglyceride; GPGM: glycerophosphorylgalactosylmonoglyceride.

	Condition	Distribution in %					
		DPG	Lyso-DPG	PG	GPGD	GPGM	Other
Medium	Penicillin	34.4	26.7	18.0	7.9	8.0	4.1
	Penicillin, -Tween 80	38.5	25.1	15.7	9.0	6.8	3.5
	No penicillin	41.9	20.0	20.7	4.6	6.3	2.9
Cells	Penicillin	10.2	33.2	14.6	10.8	14.2	3.0
	Penicillin, -Tween 80	16.0	34.2	14.0	10.2	9.2	3.1
	No penicillin	21.1	43.1	16.4	5.2	7.5	3.2

The labeled medium lipids of both the inhibited and the control culture had a high diphosphatidylglycerol content. A marked difference in the composition of labeled medium phospholipids and cellular phospholipids was in the fraction of lyso-derivatives of diphospha-

tidylglycerol. The lyso-derivatives of diphosphatidylglycerol were present in a smaller percentage in the medium lipids than in the cellular lipids. Glycerolphosphorylgalactosyldiglyceride in both medium lipids and cellular lipids was lower for the control culture than for both inhibited cultures.

b. ^{32}P-labeled lipids

The experiments with [^{14}C]oleic acid labeled lipids were repeated with ^{32}P-labeled lipids. In this experiment inhibitors of peptidoglycan synthesis were studied. Only the effects on the phospholipid composition were determined (Table XII).

After normal growth of ^{32}P-labeled cells in fresh, non-radioactive medium a relatively small amount of ^{32}P-labeled lipids was found in the culture medium. This result agrees with that described for [^{14}C]oleic acid-labeled lipids. There was a significant increase in the amount of ^{32}P-labeled lipids in the medium after peptidoglycan synthesis was inhibited. This increase was at largest in the presence of penicillin G, vancomycin and during omission of N-benzoyl-D-glucosamine from the culture medium. The medium phospholipids contained all components present in the cells. The medium phospholipids contained a relatively high amount of diphosphatidylglycerol. In the lipids of the inhibited cultures the fraction of diphosphatidylglycerol exceeded that of the ^{32}P-lipids of the control culture. The fraction of glycerophosphorylgalactosyldiglyceride and of glycerophosphorylgalactosylmonoglyceride in the medium lipids of the inhibited cultures was significantly lower than in the medium lipids in those of the normal culture. The cells showed after 3 h incubation an increased content of [^{32}P]diphosphatidylglycerol, when peptidoglycan biosynthesis was inhibited. This increase in diphosphatidylglycerol content was only small in the presence of bacitracin. We also found that the content of lyso-derivatives of diphosphatidylglycerol was higher than in the normal cells. During inhibition of peptidoglycan biosynthesis the glycerophosphorylgalactosyldiglyceride and the glycerophosphorylgalactosylmonoglyceride fraction in the phospholipids decreased in general.

TABLE XII. ^{32}P-PHOSPHOLIPID COMPOSITION OF CELLS AND MEDIUM AFTER INHIBITION OF PEPTIDOGLYCAN BIOSYNTHESIS

Cells were cultivated for 10.5 h in the presence of |^{32}P|orthophosphate (*3.2.5*). The cells were washed with non-radioactive medium, resuspended in fresh, non-radioactive culture medium (pH 6.8) containing human milk and incubated in the presence or absence of antibiotics for 3 h at 37° in a N_2/CO_2 atmosphere. Peptidoglycan biosynthesis was also inhibited by omitting human milk. After centrifugation lipids were extracted from the supernatant according to Bligh and Dyer (1959) and from the cells as described in *3.2.8*. The total radioactivity in the lipids of cells and medium was determined by liquid scintillation counting. The ^{32}P-phospholipids were analyzed by thin-layer silica gel chromatography as described in *3.2.7*. The radioactivity of each component was expressed as percentage of the total radioactivity in the ^{32}P-phospholipid components.

Abbreviations: DPG: diphosphatidylglycerol; lyso-DPG: lyso-derivatives of diphosphatidylglycerol; PG: phosphatidylglycerol; GPGD: glycerophosphorylgalactosyldiglyceride; GPGM: glycerophosphorylgalactosylmonoglyceride.

	Total activity (cpm x 10^{-4})		Distribution in %									
			DPG		Lyso-DPG		PG		GPGD		GPGM	
	cells	medium	cells	medium	cells	medium	cells	medium	cells	medium	cells	medium
Normal (0 h)	321	--	11	--	4	--	17	--	39	--	27	--
Normal (3 h)	317	4.4	15	26	6	11	7	11	34	21	34	30
Penicillin G	598	372	48	46	14	11	9	13	13	14	11	11
Vancomycin	470	317	39	42	12	11	10	16	20	17	15	11
D-cycloserine	465	29	42	43	12	14	11	13	16	11	15	14
Bacitracin	291	46	18	43	8	13	6	13	31	13	35	13
Without human milk	545	49	42	44	12	14	8	10	17	15	17	14

These results confirm the results obtained with [^{14}C]oleic acid-labeled cells. During inhibition of cell wall peptidoglycan synthesis there was a marked flux of lipids from the cell to the culture medium. These lipids comprised glycolipids and phospholipids which are characteristic membrane lipids. The glycolipid and phospholipid components that were found in the culture medium were not qualitatively different from the membrane lipid components.
The composition of the excreted ^{32}P-lipids was almost the same for all inhibitors with the exception of bacitracin. The composition of the ^{32}P-lipids excreted during inhibition of peptidoglycan synthesis resembled the composition of the ^{32}P-cellular lipids after 3 h, except in the case of bacitracin.
We cannot draw direct conclusions about the quantitative composition of the excreted lipids, since our measurements were performed under chase conditions and the labels used are only specific for a part of the lipid components: the fatty acid or the phosphate moiety.
Since the metabolic stability of these lipid moieties might vary for different components, the distribution of the label over the components does not represent the quantitative lipid composition.

3.4. DISCUSSION

3.4.1. Turnover of the cell wall

During normal growth no significant turnover of glucosamine in the cell wall peptidoglycan of *B. bifidum* var. *pennsylvanicus* was observed. However, turnover of peptidoglycan has been observed in a number of organisms. *L. acidophilus* showed during logarithmic growth a turnover of 30% per generation time (Boothby et al., 1973). Extensive turnover during logarithmic growth was observed in the peptidoglycan of *B. subtilis*, where the rate of turnover was 50% per generation time.

In *B. megaterium* during logarithmic growth a turnover rate of 30% per generation time was apparent (Mauck et al., 1971). Wong et al. (1974) showed a turnover of 15% per generation time for the peptidoglycan of *S. aureus*. Turnover of peptidoglycan was not observed in *E. coli* (Van Tubergen and Setlow, 1961), *S. faecalis* (Boothby et al., 1973), and in a diaminopimelic acid-requiring mutant of *B. megaterium* during vegetative growth (Pitel and Gilvarg, 1970).
Lack of turnover might be correlated with the length of the pulse-label period and the time of measurement. In *L. acidophilus*, cells labeled during 6 generations with a peptidoglycan precursor showed a lag period of 0.8-2 generation times before turnover became apparent. When the pulse period was smaller than 0.2 generation time no turnover was found for at least 2 generation times (Boothby et al., 1973). Mauck and Glaser (1970) found that in *B. subtilis* freshly synthesized peptidoglycan was not subject to turnover.
In our experiments the label was added during a period corresponding to 2/3 generation time whereas the course of radioactivity was studied over a period of maximally 1 generation time. Possibly the glucosamine incorporated in our experiments is not subject to turnover for a certain time in analogy to the examples mentioned.
We did also not find a significant turnover of peptidoglycan when the synthesis of protein and cell wall peptidoglycan were inhibited or when Mg^{2+} and sodium acetate were omitted from the culture medium. This demonstrated that there is under these conditions no significant loss of peptidoglycan caused by autolytic enzymatic activity. However, it is still theoretically possible that turnover of peptidoglycan occurs but that the degradation products are completely reutilized.

3.4.2. *Turnover of glycine and fatty acids in the total cells and in the cytoplasmic membrane*

No significant turnover could be observed for glycine in the total cellular protein during normal growth, during inhibition of protein and cell wall peptidoglycan synthesis and during omission of culture components. The results also showed that there are no extensive losses of protein from the cell under these conditions due to proteolytic breakdown.

During normal growth no turnover of oleic acid and palmitic acid in the cellular lipids was apparent. Since turnover of lipids in bacteria is not uncommon it may well be that under such conditions there still occurs turnover with complete reutilization of the fatty acyl group. In this way the total radioactivity is conserved during growth.
A marked turnover occurred in cells of which cell wall peptidoglycan synthesis was inhibited. The turnover phenomena for oleic acid during inhibition of cell wall biosynthesis could be reproduced with palmitic acid as label, indicating that the phenomenon is not specific for oleic acid-labeled lipids. It is remarkable that the increased turnover is observed in those cases in which the incorporation of oleic acid and palmitic acid was increased compared to normal cells.

The parameters used in our study of membrane turnover do not allow us to describe turnover in absolute terms. From the data obtained we can draw still some useful conclusions.
During normal growth, during inhibition of protein synthesis and during omission of Mg^{2+} and sodium acetate from the culture medium the amount of [^{14}C]oleic acid label per mg membrane protein was almost constant. However, during inhibition of peptidoglycan synthesis there was a significant decrease of [^{14}C]oleic acid label per mg membrane protein which must be ascribed to an increase in turnover. This increased turnover corresponded to the observed turnover of the total lipids during inhibition of peptidoglycan synthesis. Actinomycin-D inhibited cells were an exception, but since the turnover of the total lipids was rather small, it is possible that the accuracy of measurement of turnover in the membrane was not sufficient to detect an increased turnover in the membrane lipids.
The results found for the membrane lipid turnover indicated that the increase in lipid turnover rate, after inhibition of cell wall synthesis, is a process in which the membrane itself is involved and is not only due to turnover of extramembranous (cytoplasmic) lipids.
The method used for measuring turnover gives only an indication of the oleic acid turnover in the total lipids. This method does not discriminate between the different turnover rates of individual lipid

components or between turnover of fatty acids and of fatty acid-containing components.
The data for [^{3}H]glycine turnover in the membrane suggest that there is no turnover of membrane protein during normal growth and inhibition of protein biosynthesis, while during inhibition of cell wall biosynthesis and during omission of Mg^{2+} and sodium acetate a small increase in turnover is observed. There is one exception: bacitracin caused a dramatic loss of glycine label per mg membrane protein. Selective turnover of glycine in the membrane or losses of membrane protein may be responsible.

Kahane and Razin (1969) studied the turnover of membrane lipid and membrane protein in *Mycoplasma laidlawii* with [^{3}H]oleic acid and [^{14}C]phenylalanine. The combined membrane proteins turned over at a relatively high rate (half life of 3 h), whereas the turnover of the combined membrane lipids became apparent only after a lag period of several hours after the beginning of the chase. The rate of membrane protein turnover was decreased by chloramphenicol. This shows that membrane protein turnover and membrane lipid turnover are not necessarily coupled. We also found this in cells of *B. bifidum* var. *pennsylvanicus* during inhibition of peptidoglycan synthesis. With the exception of bacitracin-inhibited cells there was a significant turnover of membrane lipids whereas the membrane proteins showed no important turnover.
The apparent high turnover rate of membrane lipids and the apparent absence of turnover in cell wall are in sharp contrast. It shows that turnover of cell wall peptidoglycan and of membrane lipids are not necessarily coupled.

3.4.3. Loss of lipids to the culture fluid

In Chapter 2 we have seen that inhibition of cell wall biosynthesis resulted in an enhanced incorporation of fatty acids into the cellular lipids. In this chapter it was shown that besides enhanced incorporation of fatty acids there was also an enhanced turnover of fatty acid-labeled lipids compared to normal cells. Lipids, especially

glycolipids and phospholipids are lost from the cell to the culture medium in large amounts. The increased incorporation of fatty acids during inhibition of cell wall biosynthesis (Chapter 2) may indicate that the fatty acids lost from the cells are replaced.
In contrast to the considerable loss of polar lipids there were no significant losses of protein and peptidoglycan from the cell. From this we can conclude that the loss of lipids is probably not due to cellular lysis. Observation of cells by phase-contrast microscopy also showed no signs of extensive lysis. Loss of cellular lipids can amount, as was shown, to 65% of the original lipid content present. If loss of lipids was due to destruction, a large fraction of the cells should have been desintegrated.

Excretion of lipids of cells during growth inhibition has rarely been reported by other investigators. Growing cultures of the Gram-negative bacteria *E. coli* and *Salmonella typhimurium* excreted phospholipids but also proteins and lipopolysaccharides into the culture medium (Rothfield and Pearlman-Kothencz, 1969). When protein synthesis was inhibited the excretion was markedly increased. The excreted components appeared to belong to a membrane complex of the outer membranes of the bacterial cell envelope. A lysine-requiring auxotroph of *E. coli*, blocked in its diaminopimelic acid decarboxylase, excreted lipoproteins and lipopolysaccharides into the culture medium when it was grown under conditions of lysine limitation. This caused a reduction of the synthetic rate of peptidoglycan (Kolenbrander, 1968). Crowfoot et al. (1972[a]) reported that the membrane phospholipids of an unsaturated fatty acid auxotroph of *E. coli* were subjected to turnover. The phospholipids were excreted into the medium and were replaced in the cell with newly synthesized phospholipids. Protein was also lost from the cells. In the absence of protein synthesis the phospholipid turnover continued. In the wild-type parent strain turnover occurred only under conditions, where protein synthesis was inhibited but lipid synthesis continued. The phospholipid, accumulating in the culture medium as a result of lipid turnover appeared to be part of a losely bound low density complex arising from the cell envelope (Crowfoot et al., 1972[b]).

Excretion of lipids into the culture medium under the influence of cell wall biosynthesis-inhibiting antibiotics was reported by Nakao et al. (1973) and Kikuchi et al. (1973). Addition of penicillin to cells of *Corynebacterium alkanolyticum*, growing on n-parafinic medium caused simultaneous excretion of phospholipids, UDP-N-acetylhexosamine derivatives and L-glutamic acid. Only penicillin and cephalosporin, and no other inhibitors of cell wall biosynthesis could induce this effect. Penicillin, added to cells in the logarithmic growth phase caused a decrease of the intracellular lipids to 50% of those of intact cells, grown in the absence of penicillin. Addition of penicillin to stationary phase cells caused no change in the cellular content of phospholipids. The excretion of phospholipids was correlated with the excretion of L-glutamic acid. The same phospholipids were excreted by the cells as were present in the cells, which was also for the fatty acids (Kikuchi et al., 1973). Addition of penicillin to cultures of *Brevibacterium thiogenalis* also caused excretion of phospholipids and fatty acids. However, no decrease in the phospholipid content of the cells was found (Kikuchi et al., 1973).

In *B. bifidum* var. *pennsylvanicus* excretion of lipids was found after inhibition of peptidoglycan biosynthesis in various ways. Antibiotics, affecting biosynthesis at different levels and also inhibition of peptidoglycan biosynthesis by nutrient depletion resulted in an increased turnover and excretion of lipids. However, the extent of the change varied for the various inhibitors applied. The change of turnover rate may be influenced by the antibiotic itself and by the extent of inhibition of peptidoglycan biosynthesis.
The phenomena may be a more general effect of inhibition of cell wall synthesis in actively growing cells. Possibly membrane synthesis continues when membrane and cell wall synthesis are uncoupled. This would result in an increase of membrane protein as well as lipids in the cell. Increase in lipid content may be corrected by excretion of lipids. This has also been shown for a lipid-overproducing mutant of *E. coli* (Holden, 1973).
In the second place one might expect that, since there is no synthesis of peptidoglycan, little damage of the cell wall may result in holes,

exposing parts of the membrane to the environment of the cells, which has a lower osmotic pressure. Parts of the membrane may then protrude through the holes and release lipids. The lipids excreted from the cells of *B. bifidum* var. *pennsylvanicus* after inhibition of peptidoglycan synthesis comprise all characteristic membrane lipids. This suggests that the membrane itself is involved in this process. It also suggests that the turnover of lipids, found in preceding experiments, is due to the turnover of entire lipid components rather than to the turnover of single fatty acids. This suggestion was confirmed by the finding of ^{32}P-labeled phospholipids in the culture medium of inhibited cultures.

The distribution of ^{14}C-label or ^{32}P-label over the different lipid components in each lipid fraction does not give direct information about the real lipid composition since it can be affected by the metabolic stability of the various lipid components and may then be influenced by the chase conditions applied in our experiments.

Van Schaik and Veerkamp (1975), who made similar studies under non-chase conditions, found that upon inhibition of peptidoglycan synthesis the lipid-phosphorous content in the cells was considerably increased and that this increase was due to a marked increase of diphosphatidylglycerol and its lyso-derivatives. Bacitracin inhibition formed an exception. Thus we may conclude that the agreement between the ^{32}P-lipid composition of cellular lipids and excreted lipids does indeed mean that the phospholipid composition of excreted lipids and cellular lipids after inhibition of peptidoglycan synthesis are about the same (with the exception of bacitracin) but that they differ from that in normal cells.

One might expect that extensive losses of lipids during inhibition of peptidoglycan synthesis affect the lipid content and lipid composition of the cells. We have seen that under these conditions there was also an increased synthetic activity for lipids in these cells. We do not know whether the loss of lipids is completely compensated by an increased synthetic activity. We shall study this matter in Chapter 4.

3.4.4. Consequences of the turnover studies

The experiments described in this chapter have a number of consequences for the interpretation of the incorporation data for cell wall and cell membrane precursors (Chapter 2).

(i) *Synthesis of peptidoglycan during inhibition of protein biosynthesis and during omission of sodium acetate and Mg^{2+} from the culture medium.* We did not find a significant turnover of glucosamine in the cell wall peptidoglycan. This means either that no turnover or turnover of glucosamine with a complete reutilization of the turnover product occurs. In both cases incorporation of glucosamine molecules into the peptidoglycan reflects synthesis of new polymers rather than replacement of glucosamine molecules. We found that in the absence of protein biosynthesis and during omission of Mg^{2+} and sodium acetate there was a significant incorporation of glucosamine molecules. The absence of an apparent turnover suggests that this is due to a net synthesis of peptidoglycan.

(ii) *Synthesis of lipids during inhibition of protein biosynthesis with chloramphenicol and tetracyclin and during omission of sodium acetate and Mg^{2+} from the culture medium.* There was a significant incorporation of oleic acid per mg membrane protein during inhibition of protein synthesis. However, when protein biosynthesis was inhibited we could observe no change in the amount of [^{14}C]oleic acid per mg membrane protein. This suggests that the incorporation of oleic acid into the membrane lipid represents net synthesis rather than replacement of oleic acid molecules.

The same conclusion is valid for the total cellular lipids. During inhibition of protein biosynthesis by chloramphenicol and tetracyclin there was no apparent turnover of oleic acid whereas there was a significant incorporation of oleic acid. This reflects a net synthesis of lipids. During omission of Mg^{2+} and sodium acetate from the culture medium there was a significant incorporation of oleic acid into the membrane per mg membrane protein, whereas there was no significant

change of [^{14}C]oleic acid per mg membrane protein. This suggests that during omission of Mg^{2+} and sodium acetate there is a net synthesis of lipids.

(iii) *Synthesis of lipids during inhibition of peptidoglycan synthesis.* We found during inhibition of peptidoglycan synthesis an increased incorporation of radioactivity into lipids. However, there was also an increased turnover of lipids. The increased incorporation may be related to the increased turnover of lipids. It is not possible to conclude from these experiments whether the net result of lipid turnover and lipid synthesis is positive or negative.

CHAPTER 4

INFLUENCE OF ANTIBIOTICS AND NUTRIENT DEPLETION ON THE COMPOSITION AND PROPERTIES OF THE MEMBRANE

4.1. INTRODUCTION

In the preceding chapters we described the effects of growth inhibition on synthetic processes, involved in membrane and cell wall formation. It was shown that during inhibition of the synthesis of membrane proteins that of the membrane lipids continued, while during inhibition of cell wall synthesis the synthesis and turnover of membrane lipids increased. The information obtained is limited, since only relative rates were studied. Changes in the composition and properties of the membrane were not considered. In this chapter some information on this point will be given.

Glycolipids and phospholipids are the predominant lipids of the membrane. In order to study the effects of growth inhibition on the lipid content, the lipid-galactose and lipid-phosphorus content of the cells were determined. The fatty acid composition of the membrane lipids may have important consequences for the physical and biochemical properties of the membrane. For this reason the influence of growth inhibition on the fatty acid composition was determined. In addition to the membrane lipid composition, the qualitative protein composition and the amino acid composition of membranes from normal and inhibited cells were studied.

The osmotic behavior of the protoplasts can reflect some properties of the membrane. We studied this behavior in connection with the permeability for Rb^+ ions and with the Na^+ and K^+ contents of the cells.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

The chemicals used are described in 2.2 and 3.2 unless otherwise mentioned. ^{86}RbCl was obtained from the Radiochemical Centre, Amersham, England.

4.2.2. Extraction of lipids

Cells from 100 ml of culture were harvested by centrifugation and washed two times with 0.2 M acetate buffer (pH 5.0) of 0°. After suspension in 50 ml 0.2 M acetate buffer (pH 5.0), extraction of lipids was carried out according to Bligh and Dyer (1958). The cell residue was extracted three more times with 25 ml of chloroform-methanol (2:1, by vol). Both extracts were combined and intensively mixed with 50 ml chloroform and 50 ml distilled water. The mixture was allowed to separate into two distinct phases. The organic phase was collected. The aqueous phase was extracted once with 50 ml chloroform. The solvent was evaporated *in vacuo* and the lipids were redissolved in chloroform-methanol (2:1, by vol).

4.2.3 Determination of lipid-phosphorus and lipid-galactose

Phosphorus in lipid extracts was determined in triplicate by the procedure of Bartlett (1958) and galactose with the anthrone method described by Radin et al. (1955) in fivefold.

4.2.4. Determination of DNA and RNA

DNA was determined according to the procedure of Burton (1955). Samples of 10 ml were removed from the culture and 0.22 ml 70% $HClO_4$ in water was added. After 2 h at 0° the precipitate was centrifuged in the cold and resuspended in 3 ml water. Then 0.75 ml 1 M $HClO_4$ was added and the mixture was placed for 30 min at 4°. After centrifugation during 15 min at 4° the precipitate was twice extracted at 90° with

4 ml 0.5 M $HClO_4$. These extracts were combined and 2 ml extract was mixed with 0.1 ml 0.07 M ethanol and 4 ml diphenylamine reagent (1.5 g diphenylamine + 1.5 ml 98% H_2SO_4 per ml glacial acetic acid). The absorbance at 600 nm was measured after 16 h at room temperature. Solutions of calf thymus DNA were used as standard.
RNA was determined according to Ogur and Rosen (1950). Samples of 1 ml were removed from the culture and mixed with 1 ml 10% TCA of 0°. The mixture was kept at 0° for 1 h and centrifuged in the cold. The pellet was twice resuspended in 5 ml 5% TCA and centrifuged in the cold. The sediment fraction was resuspended in 3 ml 1 M $HClO_4$ of 4° and the suspension was kept at 4° overnight. The absorbance (260 nm) of the supernatant after centrifugation was measured. The absorbance of a solution containing 1 mg/ml RNA was assumed to be 28.0 (Gale and Folkes, 1953).

4.2.5. Isolation of membrane lipids for fatty acid analysis

Lipids were extracted from 100 ml culture as described in *4.2.2.* After evaporation of the solvents under nitrogen, the lipids were dried over P_2O_5 under a nitrogen atmosphere. The dried lipids were redissolved in chloroform and applied on columns of 3 g silicic acid in chloroform. Lipids were eluted with successively 50 ml chloroform and 50 ml chloroform-methanol-water (10:10:1, by vol). The latter fraction was collected and the solvent was evaporated under nitrogen.

4.2.6. Preparation of fatty acid methyl esters

Fatty acid methyl esters were prepared by methylation of about 5 mg of lipids in 0.5 ml n-hexane with 1 ml BF_3-methanol (10% in methanol) under nitrogen gas at 100° during 10 min. After the methylation 2 ml water and 4 ml n-pentane were added and the reaction mixture was intensively mixed. After separation into two phases the pentane phase was collected and once washed with water. The fatty acid methyl esters were stored in pentane at -20° under nitrogen.

4.2.7. Quantitative analysis of fatty acid composition

Columns of 0.16 inch x 6 ft of 15% diethyleneglycolsuccinate on 60-80 mesh Gas-chrom P were used at 168° in a Packard gas chromatograph model 7821. The fatty acids were identified by comparison of the relative retention volume of methyl esters with those of standard methyl esters of saturated, unsaturated, iso- and anteiso fatty acids (Veerkamp, 1970). The relative composition of fatty acid mixtures was determined by measuring the area under the peaks. The area under a peak was calculated by multiplication of peak height with the width at half height (Veerkamp, 1970).

4.2.8. Preparation of the cytoplasmic membrane fraction

Cells from 100 ml cultures were harvested by centrifugation at 10 000 g at 4° and washed twice with 0.1 M Tris-HCl (pH 6.8). Lysozyme was added in a concentration of 0.6 mg/ml suspension. The suspension was incubated during 40 min in a water bath of 37°. Protoplast formation was controlled by phase-contrast microscopy. The protoplasts were lysed completely by sonication 4 times during 15 sec while cooling in ice. Desoxyribonuclease was added in a concentration of 0.5 µg/ml lysate and the lysate was incubated for 5 min in a water bath of 37°. The completeness of desintegration of the protoplasts was checked by phase-contrast microscopy. The lysate was centrifuged at 100 000 g for 30 min at 4°. The sediment fraction was washed four times with 20 mM Tris-HCl (pH 5.0) containing 5 mM $MgSO_4$ and once with distilled water. The sediment fraction was finally taken up in distilled water, freeze-dried and stored at -20°.

4.2.9. Amino acid analysis

About 5 mg membrane preparation were hydrolyzed in 1 ml 6 N HCl (suprapur, Merck, Darmstadt) in sealed, evacuated glass tubes at 110° for 16 h. Norleucine was used as an internal standard. The hydrolyzate was filtered and the filtrate was evaporated *in vacuo* at 40°. The residue was dried *in vacuo* over NaOH pellets. The amino acid com-

position of the hydrolyzate was determined on a Beckman Multichrom Liquid Column Chromatograph model 4255 using a single column system.

4.2.10. Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out according to the method of Laemmli (1970). Gels were prepared by mixing 5 ml stock solution containing 30% acrylamide and 0.8% bisacrylamide with 7 ml 1 M Tris-HCl (pH 8.8) and 25 µl N,N,N',N',-tetramethylethylenediamine. The volume was adjusted to 18 ml with distilled water, whereupon the solution was deaerated. Then 0.47 ml 1% $K_2S_2O_8$ and 0.18 ml 10% sodium dodecylsulphate (SDS) were added. Glass tubes, siliconized by treatment with 2% dichlorodimethylsilane in carbontetrachloride, were each filled with 1.25 ml of the mixture. This was overlayered with about 0.2 ml 0.1% SDS-solution. Polymerization was allowed to take place at room temperature overnight. After removing the SDS-solution a stacking gel was placed on top of the gel. The solution for the stacking gel consisted of 1 ml 30% acrylamide, 0.8 ml 0.8% bisacrylamide, 2.5 ml 1 M Tris-HCl (pH 6.8) and 0.13 ml N,N,N',N',-tetramethylethylenediamine. After mixing these contents the volume was adjusted to 7.5 ml with distilled water, the mixture was deaerated and 1 ml 1% $K_2S_2O_8$ and 0.1 ml 10% SDS were added. 75 µl of stacking gel solution was layered on top of the gel. The stacking gel itself was overlayered with 0.2 ml 0.1% SDS-solution. The gel was then allowed to polymerize for 15-30 min at room temperature.
Membrane samples were prepared by suspending 5-7 mg membrane preparation in 1 ml distilled water. To this solution were added: 0.5 ml 4% SDS, 20% glycerol in water, 0.5 ml 0.125 M Tris-HCl (pH 6.8), 1 mg 1,4-dithioerythrit and 0.4 ml 0.08% bromophenol blue in water. The gel tubes were placed in a "Polyanalyst" gel electrophoresis apparatus. The buffer solution for both upper and lower reservoirs of the apparatus contained 3 g Tris, 14.4 g glycine and 1 g SDS per 1 water (pH 8.6). A 50 µl sample was applied to the stacking gels. Electrophoresis was carried out at room temperature with a constant current of 1 mA per tube, using the bottom electrode as anode. The gels were stained after electrophoresis with a 0.2% Coomassie

Brilliant Blue R solution in 50% methanol and 7% acetic acid. The gels were destained with 7% acetic acid and 5% methanol at 60°.

4.2.11. Determination of the osmotic stability of protoplasts

Cells from 10 h-cultures were harvested and resuspended in an equal volume of fresh culture medium (pH 6.8). The suspension was divided into two batches of equal volume. One of the batches received antibiotics, the other batch served as a control culture.
In experiments in which omission of magnesium and human milk was studied, cells were resuspended in medium lacking these components. Both batches were incubated for 2.5 h (in some experiments 5 h) at 37° in N_2/CO_2 atmosphere. Cells were harvested by centrifugation at 4°, washed twice with 0.1 M phosphate buffer (pH 6.8) containing 10 mM Mg^{2+}, and finally suspended in the same buffer containing 0.7 M sucrose. All sucrose-containing solutions were prepared immediately before use. To the suspension of inhibited cells antibiotics were added in the same concentration as during the incubation at 37°. Lysozyme was added in a concentration of 1 mg/ml and cells were incubated at 37° for 30 min. Protoplast formation was controlled by phase-contrast microscopy. Immediately after the preparation of protoplasts, 0.2 ml of protoplast suspension was pipetted to 5 ml 0.1 M phosphate buffer (pH 6.8) containing 10 mM Mg^{2+} and 0.7 M sucrose. The 550 nm absorbance of the suspension was measured. Then 0.2 ml protoplast suspension was pipetted in duplicate into 5 ml 0.1 M phosphate buffer (pH 6.8) containing 10 mM Mg^{2+} and a variable concentration of sucrose (0-0.7 M). Another 0.2 ml sample of protoplast suspension was added to 5 ml 0.1 M phosphate buffer (pH 6.8) containing 10 mM Mg^{2+} and the protoplasts were lysed by sonication. The suspensions were intensively mixed and left at room temperature for 30 min.
The 550 nm absorbance (A550) was corrected by subtracting the absorbance at 550 nm of the sonicated protoplast suspension (A550 son). The protoplast suspensions were centrifuged for 15 min at 15 000 g (4°) and the supernatants were collected. The 260 nm absorbance of

the supernatant (A260) was determined after 10-fold dilution with water. The lysis was expressed as A260/A260 son, where A260 son is the absorbance at 260 nm of the supernatant of the sonicated protoplast suspension. Curves were plotted of (A550-A550 son) and of (A260/A260 son x 100) versus the molarity of the sucrose in the test buffer. The 50% lysis points from both curves were used as a measure of the osmotic stability.

The absorbance of cell and protoplast suspensions was measured at 550 nm with a Bausch and Lomb Spectronic 20 spectrophotometer using 13 mm round bottom glass cuvettes.

UV-absorbing material was measured at 260 nm with a Zeiss PM Q II spectrophotometer in 1 cm quartz cuvettes.

Protoplast suspensions were sonicated during 30 sec in ice using a Branson B-12 Sonifier, equipped with a microtip. A maximal energy output (65 W) was used.

The osmotic pressure of sucrose-phosphate buffer solutions was determined on a freezing point osmometer (Advanced Instruments Inc. Needham Heights, Mass. U.S.A.) which was calibrated to give a read-out in milliosmoles per kg of solvent.

4.2.12. Measurement of the $^{86}Rb^{+}$ efflux from the cells

Cells were cultivated for 10 h in medium containing 0.088 µCi/ml ^{86}RbCl. The cells were harvested by centrifugation at 10 000 g for 15 min and the pellet of 10 ml culture was resuspended in 1 ml of the supernatant (suspension A). Then 0.25 ml of suspension A was mixed with 4.75 ml fresh, non-radioactive culture medium of 37°, containing antibiotics. A control experiment was carried out using culture medium without antibiotics. After appropriate time intervals at 37° cells from 0.25 ml of the suspension were collected by filtration through membrane filters (Selectron, BA, effective diameter 0.45 µm), and washed with 5 ml 0.1 M Na-phosphate buffer (pH 6.8). Residual radioactivity on the filter was determined by liquid scin-

tillation counting after immersing the filter in 1 ml water and 10 ml Bray scintillation fluid (Bray, 1960). The intracellular radioactivity originally present was determined from 25 µl of suspension A, using the same method as described above.

4.2.13. Measurement of the $^{86}Rb^{+}$ uptake by the cells

Cells were cultivated for 10 h and harvested by centrifugation at 10 000 g during 15 min. The pellet of a 10 ml culture was resuspended in 1 ml Norris medium, in which K_2HPO_4 was replaced by Na_2HPO_4 (K^+-poor medium). The K^+ content of this medium was about 3 meq/l. Then 0.25 ml of the suspension was mixed with 4.75 ml K^+-poor medium, containing 1.42 µCi $^{86}RbCl$ and an antibiotic. A control experiment was carried out simultaneously in medium without antibiotic. The experiments were performed at 37° under aerobic conditions. After appropriate time intervals, cells in 0.25 ml suspension were collected by membrane filtration and the radioactivity on the filter was determined as described (*4.2.12*).

4.2.14. Determination of Na^+ and K^+ contents of the cells

Fourty ml of culture was centrifuged at 20° for 15 min at 12 000 g. The cells were washed twice with 40 ml 0.25 M $MgCl_2$ (pH 5.5) and finally resuspended in 5 ml redistilled water. Concentrated HNO_3 (1 ml) was added to aid in disrupting the cells and to effect the release of intracellular cations. The suspensions were then heated in a boiling-water bath for 10 min. The volume was brought up to 50 ml with redistilled water and samples were analyzed for Na^+ and K^+ with an Eppendorf flame photometer. Na^+ and K^+ standards in the same concentration were used for calibration.

4.3.1. The lipid-phosphorus, lipid-galactose, DNA and RNA contents of cells after normal growth and after growth inhibition

Cells, grown for 10 h, were resuspended in fresh culture medium and incubated for 3 h in the presence and absence of antibiotics and in a medium from which human milk was omitted.

In Chapter 2 we reported that lipid synthesis continued in the absence of protein biosynthesis and that there was an increased synthesis and turnover of membrane lipids during inhibition of peptidoglycan synthesis. The lipid-phosphorus and lipid-galactose contents of the cells were determined in order to detect changes in the membrane lipid content of the cells. The DNA and RNA contents of the cells were also determined. The 550 nm absorbance of the cultures was recorded as a measure of the number of cells present.

The lipid-phosphorus, DNA and RNA contents increased markedly during normal growth, whereas the lipid-galactose content remained about the same (Table XIII). The results obtained for inhibited cells can be compared with those for normal cells after 3 h incubation. In general there was a decrease of the lipid-phosphorus content in the cells during incubation with antibiotics, which inhibit protein synthesis. This difference was marked during incubation with chloramphenicol. The lipid-galactose content increased significantly above that in normal cells. The DNA content decreased or remained almost the same (tetracyclin) compared to normal cells, but there was a significant increase of the RNA content during incubation with chloramphenicol and tetracyclin.

The lipid-phosphorus content of the cells tended to increase more during inhibition of peptidoglycan synthesis than in normal cells. We did not find significant deviations of the lipid-galactose content from that in control cells, except after inhibition with bacitracin, where the cells had a relatively high lipid-galactose content. The DNA content was of the same order after inhibition of peptidoglycan synthesis, but increased after D-cycloserine action.

The RNA contents became lower during incubation with penicillin and

TABLE XIII. DNA, RNA, LIPID-PHOSPHORUS AND LIPID-GALACTOSE CONTENTS OF NORMAL AND INHIBITED CELLS

Cells from 10 h-cultures were resuspended in fresh culture medium (pH 6.8) and incubated for 3 h in the presence and absence of antibiotics. The effects of omission of human milk were studied by resuspending the cells in fresh culture medium without human milk. DNA, RNA lipid-phosphorus and lipid-galactose contents are given as percentage of the contents of normal cells after 3 h incubation in fresh medium. The data are the means (± SD) of 5 experiments for DNA and RNA and 3-4 for lipid-galactose and lipid-phosphorus. For normal (3 h) cultures the contents are: DNA: 4.0 ± 0.6 mg/l; RNA: 45 ± 6 mg/l; lipid-phosphorus: 12.8 ± 3 µmol/l; lipid-galactose: 20.4 ± 0.2 µmol/l per unit of 550 nm absorbance.

	DNA	RNA	Lipid-phosphorus	Lipid-galactose
Normal 0 h	77 ± 9 xx	66 ± 15 x	70 ± 12 x	100 ± 4
Normal 3 h	100	100	100	100
Chloramphenicol	74 ± 17 x	150 ± 26 x	59 ± 11 xx	117 ± 11 x
Tetracyclin	92 ± 13	171 ± 32 xx	82 ± 6 x	134 ± 18 x
Actinomycin-D	63 ± 4 xx	94 ± 14	77 ± 25	122 ± 8 x
Penicillin	83 ± 10 xx	59 ± 12 xx	122 ± 19	98 ± 12
Bacitracin	99 ± 14	86 ± 16 (x)	115 ± 11 (x)	128 ± 12 x
D-cycloserine	127 ± 7 xx	117 ± 25	126 ± 4 xx	101 ± 14
Vancomycin	92 ± 13	63 ± 18 x	134 ± 17 x	99 ± 7
Without milk	102 ± 17	114 ± 17	119 ± 3 xx	100 ± 19

(x): $0.05 < p < 0.10$ (student-t test)

x : $0.01 < p \leq 0.05$

xx : $p \leq 0.01$

vancomycin. No significant deviations of the RNA content were observed after other ways of cell wall inhibition.

4.3.2. Changes in fatty acid composition of membrane lipids during growth inhibition

The influence of a relatively short period of growth inhibition (3 h) on the fatty acid composition of glycolipids and phospholipids was studied. Glycolipids and phospholipids were analyzed together, because there are no large differences in their fatty acid composition (Veerkamp, 1970). In all experiments inhibited cells and cells incubated without inhibitors were compared during the same incubation period.

Table XIV shows the fatty acid composition of glycolipids and phospholipids of cells from 10 h-cultures after 3 h growth in fresh medium.

TABLE XIV. FATTY ACID COMPOSITION IN NORMAL CELLS

Cells were cultivated for 10 h at 37°. After resuspension in fresh culture medium (pH 6.8), containing N-benzoyl-glucosamine, the cells were further incubated for a period of 3 h. Lipids were extracted and the fatty acid composition of glycolipids and phospholipids were determined. The fatty acid composition is expressed as the percentage of the total fatty acids. The results are the means of 9 experiments with their standard deviation.

Fatty acid	
12:0	0.8 ± 0.2
iso-14:0	0.9 ± 0.2
14:0	3.6 ± 0.5
16:0	29.9 ± 3.0
16:1	4.3 ± 0.5
18:0	19.3 ± 1.3
18:1	35.9 ± 2.9
18:2	1.2 ± 0.2
cyclo-19:0	3.6 ± 0.7

TABLE XV. CHANGES IN FATTY ACID COMPOSITION AFTER INHIBITION OF GROWTH

Cells were cultivated for 10 h at 37°. After resuspension in fresh culture medium (pH 6.8), containing N-benzoyl-glucosamine, the cells were further incubated with and without antibiotics. For the study of N-benzoyl-glucosamine omission cells were resuspended in culture medium without this growth factor. In the case of Mg^{2+} and sodium acetate omission cells were cultivated for 16 h. All experiments included appropriate control cultures. Lipids were extracted and the fatty acid composition of the glycolipids and phospholipids were determined. The values for the fatty acid composition are given relative to those of the normal cells, set at 100%. The results are the means of the indicated number of experiments with their standard deviation.

	Number of experiments	Fatty acid		
		16:0	18:0	18:1
Normal 3 h	-	100	100	100
Chloramphenicol	2	99 ± 8	107 ± 2	104 ± 4
Tetracyclin	1	105	106	100
Actinomycin-D	3	93 ± 8	104 ± 5	112 ± 7
Penicillin G	3	87 ± 3 x	58 ± 12 x	130 ± 6 x
Vancomycin	3	85 ± 12	75 ± 9 x	129 ± 15 (x)
D-cycloserine	4	88 ± 11	80 ± 11 x	119 ± 17
Omission of N-benzoyl-glucosamine	2	96 ± 5	92 ± 7	107 ± 1
Bacitracin	3	112 ± 3 x	77 ± 9 x	96 ± 1 x
Staphylococcin	2	106 ± 18	84 ± 1	87
Omission of Mg^{2+}	2	79 ± 25	87 ± 11	128 ± 15
Omission of sodium acetate	3	78 ± 16	77 ± 34	125 ± 4 xx

(x): $0.05 < p < 0.10$ (student-t test)

x : $0.01 < p \leq 0.05$

xx : $p < 0.01$

Hexadecanoic, octadecanoic and octadecenoic acid constitute the major fatty acids. Since the variations in the minor components are relatively small, we show only the effects of growth inhibition on changes in the content of the three major fatty acids (Table XV).
Inhibition of protein synthesis by chloramphenicol, actinomycin and tetracyclin caused only slight changes in the fatty acid composition. However, inhibitors of peptidoglycan synthesis, penicillin and vancomycin gave rise to an increase of octadecenoic acid with a concomitant decrease of octadecanoic acid. Omission of N-benzoyl-glucosamine resulted only in small changes in the fatty acid composition. The slight effects might be caused by the short incubation time. The main effect of bacitracin was decreasing the octadecanoic acid and increasing the hexadecanoic acid content. Staphylococcin decreased the octadecanoic and octadecenoic acid contents. D-cycloserine caused decrease of the octadecanoic acid content.
We studied the effect of omission of Mg^{2+} and sodium acetate on the fatty acid composition. Since the cells could be cultivated in Mg^{2+}-poor medium and medium lacking sodium acetate we preferred to study the influence of 16 h growth in these media rather than the influence of a relatively short period of incubation. More marked differences in the fatty acid composition might be expected in this way. The cells cultivated in the depleted media showed a significant increase in octadecenoic acid compared with cells from normal 16 h-cultures.
In connection with the data shown in Table XV, it is important to know the influence of differences in growth phase between inhibited cells and normal cells. An inhibited culture grows slowly or not, which might cause a phase difference between the inhibited cells and their controls. Differences in growth phase can have a marked effect on the fatty acid composition of the lipids (Veerkamp, 1969). Ageing of cultures of *B. bifidum* var. *pennsylvanicus* caused a decrease of hexadecanoic acid accompanied by an increase of octadecanoic and octadecenoic acid. The average chain length increased during ageing. The effects in our study of the inhibitors, penicillin, vancomycin and after growth in Mg^{2+}-poor and sodium acetate-depleted medium might in part be caused by a process of ageing (increase of octadecenoic acid, but the decrease of octadecanoic acid is not in

agreement with this. The effects of bacitracin and staphylococcin also conflict with a process of ageing, since the octadecenoic acid content decreased. Another difference between control cultures and inhibited cultures, which might possibly affect the fatty acid composition, is the pH of the culture medium. However, Veerkamp (1970) showed that differences in acidity of the medium of *B. bifidum* var. *pennsylvanicus* had no significant influence on the fatty acid composition of the membrane lipids. In our experiments the differences between the pH of the control cultures and inhibited cultures never exceeded 0.2 pH unit. An exception is formed by the sodium acetate-depleted cultures, where this difference was about one pH unit. The more acid conditions towards the end of the incubation may have influenced the fatty acid composition.

4.3.3. Composition of membrane protein

We investigated whether changes in the membrane protein composition occurred upon inhibition of growth. Two aspects of the membrane protein composition were investigated: (i) the amino acid composition; (ii) the qualitative composition of membrane proteins, separated by polyacrylamide-gel electrophoresis.
The molar amino acid composition of the total membrane proteins of normal and inhibited cells (Table XVI) shows no pronounced differences due to inhibition of protein and cell wall peptidoglycan synthesis or to growth in sodium acetate- and Mg^{2+}-depleted media.
In order to obtain information about qualitative changes in the composition of the membrane proteins, the membrane fractions were analyzed by polyacrylamide-gel electrophoresis. Several systems were tested so as to obtain a good resolution of the membrane proteins. Alkaline polyacrylamide electrophoresis in the presence of 8 M urea (Panos et al., 1972) and polyacrylamide electrophoresis on acid gels (Rottem-Razin, 1967) gave poor results. However, the method of Laemmli (1970) gave a good resolution of the membrane proteins.
In Fig. 43 examples of electropherograms of membranes of normal and inhibited cells are shown. In every experiment membranes were analyzed which originated from the same mother culture. At least 33 different

TABLE XVI. AMINO ACID COMPOSITION OF MEMBRANE PREPARATIONS

Cells were cultivated for 10 h at 37° and resuspended in fresh culture medium (pH 6.8) and in medium from which human milk, sodium acetate and Mg^{2+} had been omitted. The cells were incubated for 3 h in the presence and absence of antibiotics. Membrane fractions were isolated from the cells and the amino acid composition of the membrane protein was determined. The amino acid composition is expressed in mol%. The data for control membranes are the mean of 3 experiments.

Amino acid	Cont	Peni	Baci	Van	Cyclo	Act	-HM	Chlor	$-Mg^{2+}$	-NaAc
Lysine	5.9	5.5	6.1	6.4	6.1	5.7	6.1	5.2	5.6	5.5
Histidine	2.2	2.2	2.2	2.3	2.5	2.1	2.4	1.6	1.7	1.8
Arginine	6.7	7.1	7.6	8.0	6.9	7.7	7.6	7.3	7.3	7.0
Aspartic acid	13.5	14.2	10.8	13,8	13.4	15.2	14.0	15.9	15.4	15.0
Threonine	6.2	6.0	5.8	5.9	6.1	6.0	6.2	6.2	6.3	6.3
Serine	7.0	7.3	6.9	7.1	6.8	8.3	7.1	8.0	7.6	7.3
Glutamic acid	7.8	7.1	6.9	7.1	7.7	7.5	7.2	6.8	7.0	8.0
Proline	3.8	3.7	3.6	3.3	3.6	2.8	3.5	3.0	3.2	3.6
Glycine	10.3	11.0	10.0	10.1	10.0	10.8	10.2	11.0	10.5	10.1
Alanine	11.7	11.4	10.5	11.3	11.1	10.6	11.3	11.8	11.3	11.9
Cysteine	1.2	1.4	1.2	1.2	1.0	1.6	1.4	2.3	2.0	1.8
Valine	5.6	5.0	9.5	5.3	5.6	4.8	5.2	4.6	4.8	5.0
Methionine	0.6	0.7	1.1	1.3	1.1	0.7	0.9	0.5	0.8	0.6
Isoleucine	4.2	4.3	4.4	4.1	4.5	4.0	4.0	4.1	3.8	3.8
Leucine	7.9	7.6	7.9	7.6	8.2	7.1	7.3	7.2	7.3	7.3
Tyrosine	2.5	2.5	2.6	2.4	2.4	2.2	2.4	2.0	2.3	2.4
Phenylalanine	2.9	3.0	2.9	3.0	3.0	2.9	3.0	2.5	2.9	2.8

Abbreviations: Cont - control, Peni - penicillin, Baci - bacitracin, Van - vancomycin, Cyclo - cycloserine, Act - actinomycin, -HM - depletion of human milk, Chlor - chloramphenicol, $-Mg^{2+}$ - omission of magnesium ions, -NaAc - omission of sodium acetate.

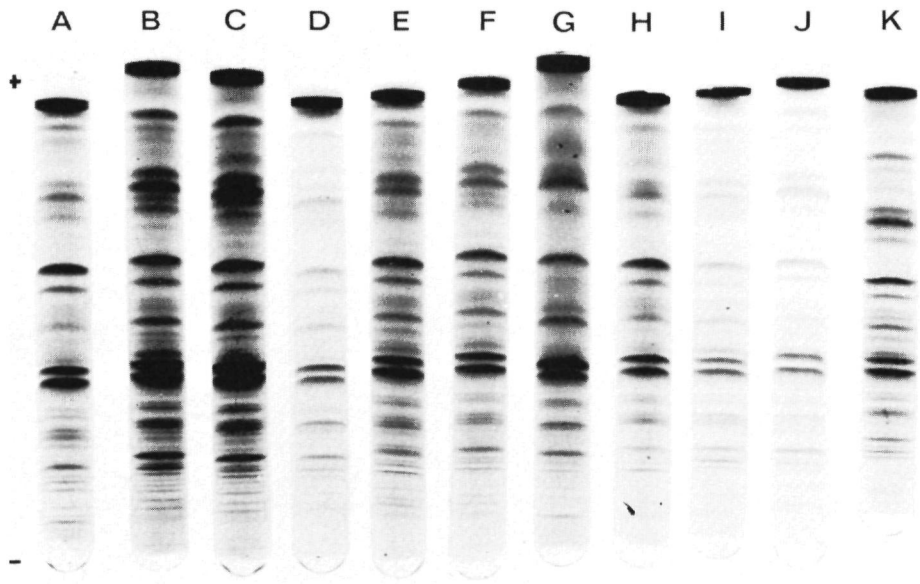

Fig. 43. Polyacrylamide-gel electropherograms of membranes. Cells were cultivated for 10 h at 37° and resuspended in fresh culture medium (pH 6.8) or in culture medium from which N-benzoyl-glucosamine, Mg^{2+} or sodium acetate were omitted. Antibiotics were added to normal media, control cells received no antibiotics. Cells were further incubated for 3 h at 37°. Membranes were isolated and polyacrylamide-gel electrophoresis was performed as described in *4.2.10*.
A: control; B: penicillin G; C: bacitracin; D: vancomycin; E: control; F: D-cycloserine; G: actinomycin-D; H: omission of human milk; I: control; J: chloramphenicol; K: omission of Mg^{2+}.

protein bands could be detected. At the top of the gels a protein fraction was found concentrated in one band, which may consist of large (lipo) protein aggregates, since this protein fraction moves relatively fast in the gel.
There were no significant qualitative differences in the electrophoretic patterns of membranes of normal cells and inhibited cells. The bands which were typical for the membranes of the control cells could also be identified in the electropherograms of the membranes of inhibited cells, although there were differences in the intensity of the bands.

4.3.4. Osmotic stability of protoplasts

Treatment of cells of *B. bifidum* var. *pennsylvanicus* with the cell wall degrading enzyme lysozyme results in solubilization of the cell wall. By this treatment the cell looses its rigidity and osmotic protection. The naked cell body (protoplast) is only surrounded by the cytoplasmic membrane and can be protected from osmotic lysis by an iso- or hypertonic environment. This environment may be constituted by solutions of substances, which do not enter the cell but decrease the extracellular water activity. In this section the response of protoplasts, derived from normal and inhibited cells of *B. bifidum* var. *pennsylvanicus*, to external osmotic forces is studied.

Cells were transformed to protoplasts with lysozyme at pH 6.8. To prevent lysis during protoplast formation a buffered 0.7 M sucrose solution was used as an osmotic stabilizer. The protoplast suspension was then diluted 25-fold in 0.1 M phosphate buffer (pH 6.8) containing sucrose in varying molarities (0-0.7 M). These solutions constituted different osmotic environments for the protoplasts (Fig. 44).
We determined the absorbance at 550 nm of the protoplast suspension in the phosphate-buffered sucrose solutions after 30 min at room temperature as a rough measure for the number of intact protoplasts. Lysis of protoplasts causes a decrease of the absorbance of the suspension. To correct the absorbance for non-protoplast contributions, the absorbance of a sonicated protoplast suspension (100% lysis) was

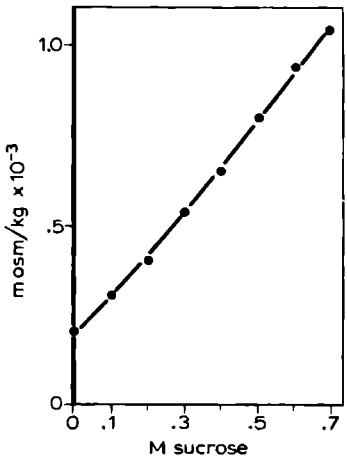

Fig. 44. Osmotic pressure of 0.1 M phosphate buffer (pH 6.8)-sucrose solutions.

subtracted. However, it is known that the absorbance of protoplast suspensions is not only dependent on the protoplast concentration, but also on the shape and size of the protoplasts (Gilby and Few, 1959). Since this might introduce inaccuracies in the determination of lysis, we also determined lysis of protoplasts from the absorbance at 260 nm of the protoplast supernatant. The absorbance at 260 nm is predominantly caused by nucleic acids, which are released during protoplast lysis. We set the absorbance of the supernatant of a sonicated protoplast suspension at 100% and expressed lysis as the percentage of maximal lysis.

The methods gave reproducible results, when cells were tested from the same original mother culture in different experiments. However, marked differences were sometimes found when protoplasts of cells from different cultures were compared. It was therefore necessary to compare the results of protoplasts from inhibited cells with the results of protoplasts of control cells from the same mother culture. We always used cells from 10 h-cultures, which were re-suspended in fresh culture medium (pH 6.8) and incubated for 2.5 h (in some cases 5 h) with and without inhibitors. During protoplast formation inhibitors used during cultivation were also present.

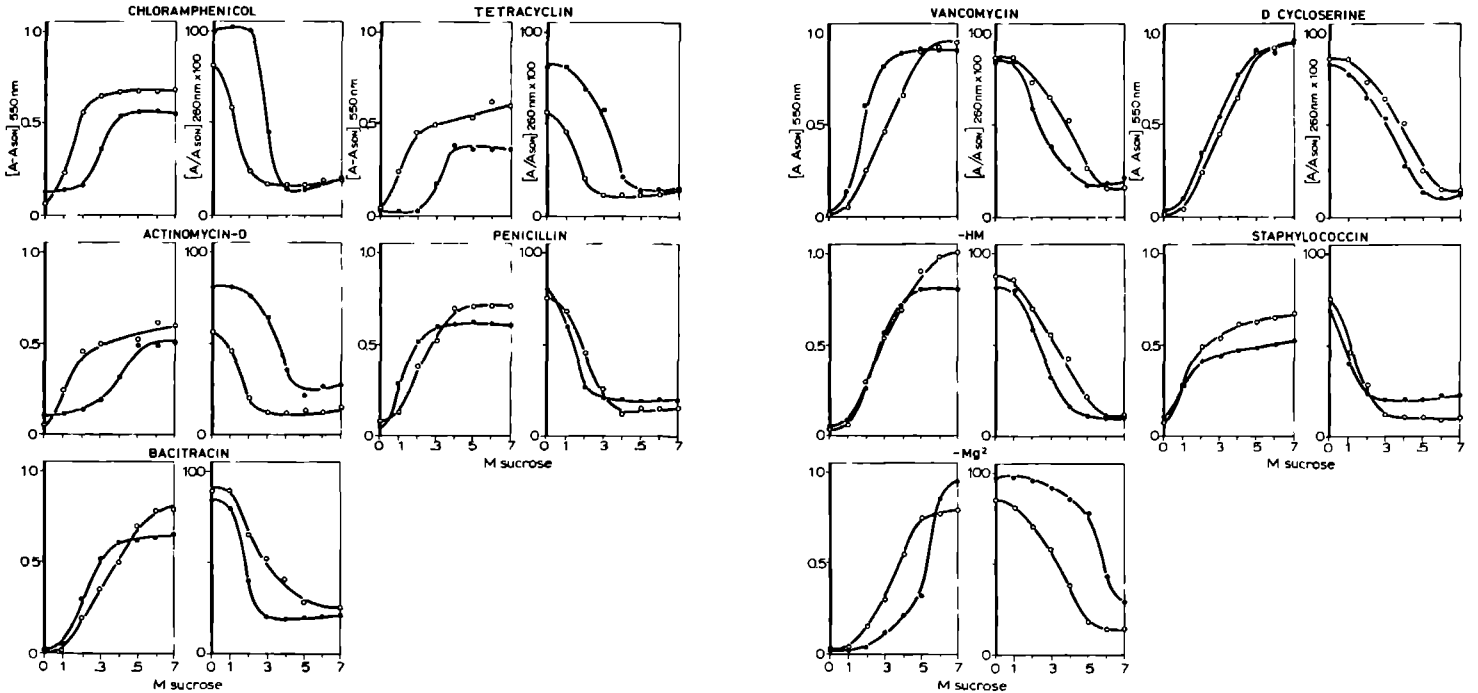

Fig. 45. Influence of growth inhibition on the osmotic behavior of protoplasts. Cells were cultivated for 10 h at 37° and incubated for 2.5 h at 37° in fresh culture medium (pH 6.8) in the presence and absence of antibiotics. Cells were also incubated in media from which N-benzoyl-glucosamine or Mg^{2+} were omitted respectively. Protoplasts were prepared from normal and inhibited cells and the osmotic behavior was determined as described in *4.2.11*. Open symbols: normal cells; closed symbols: inhibited cells. Representative experiments are given.

Fig. 45 shows plots of the corrected values for the absorbance at 550 nm of protoplas suspensions and the fraction of lysis (determined by measurements of the absorbance at 260 nm of protoplast supernatants) versus the molarity of the sucrose solutions. It is remarkable that there is no sharp transition from intact protoplasts to lysed protoplasts when the osmotic pressure of the environment is lowered. This indicates that the protoplasts are rather a population with a relatively large range in the individual osmotic properties.

The 50% lysis point (L50) was used as a parameter for the average osmotic properties of a protoplast population. This was determined from the curves in Fig. 45 as the sucrose molarity at which 50% of the transition going from a maximal to a minimal value for $(A/A\ son)_{550}$ and $(A/A\ son)_{260}$ occurred. In general there was a good agreement between the data obtained for L50 on the basis of the A_{550} measurements and of the A_{260} measurements (Table XVII). The difference between L50 for inhibited protoplasts and for control protoplasts (ΔL50) reflects changes in the average osmotic properties of protoplast populations. For $\Delta L50 > 0$ lysis in protoplasts of inhibited cells occurs at a relatively higher osmolarity of the sucrose solution than for control protoplasts. For $\Delta L50 < 0$ lysis of protoplasts from inhibited cells occurs at a relatively lower osmolarity.

Despite the large variations between the L50 points for normal protoplasts in different experiments, the effects of growth inhibitors appeared to be reproducible in a qualitative sense. Inhibition of protein biosynthesis caused that the protoplasts lysed at a relatively higher osmotic pressure than for control protoplasts. This effect was also caused by incubation of cells in a medium from which Mg^{2+} had been omitted. This experiment was performed after 5 h incubation of the cells in order to obtain fuller exhaustion of the endogeneous Mg^{2+} from the cells. However, inhibition of peptidoglycan biosynthesis (penicillin G, bacitracin, D-cycloserine, vancomycin and omission of N-benzoyl-glucosamine from the growth medium) in nearly all experiments lowered the average osmotic pressure at which the protoplasts lysed compared with control protoplasts. This effect was also found for staphylococcin action. However, during preparation of protoplasts from staphylococcin-inhibited cells there was always

a higher fraction of the protoplasts already lysed at 0.7 M sucrose. One would expect that the weak protoplasts would lyse first, while the stronger protoplasts would remain intact, which would cause a decrease of L50. Such an effect was not generally found for the inhibitors of cell wall biosynthesis. Also during preparation of protoplasts from cells inhibited by actinomycin or by Mg^{2+} omission a higher fraction of protoplasts already lysed at 0.7 M sucrose was found. This indicates that the real ΔL50 should be larger than is given in Table XVII.

4.3.5. Efflux and uptake of electrolytes during growth inhibition

In the preceding section we have shown that after inhibition of protein synthesis protoplasts lysed at a higher osmotic pressure than protoplasts from normal cells, whereas inhibition of peptidoglycan synthesis caused lysis at a relatively lower osmotic pressure. In this section experiments are described in which we tried to relate the osmotic behavior of the protoplasts with uptake or efflux of electrolytes. Intact cells, rather than protoplasts were used for this study. First the uptake and release of $^{86}Rb^{+}$ ions were studied. Leakage of $^{86}Rb^{+}$ was studied in cells cultivated for 10 h in the presence of $^{86}Rb^{+}$ and determination of the amount of $^{86}Rb^{+}$ left in the cells after incubation in fresh culture medium. The residual intracellular amount of $^{86}Rb^{+}$ was determined by membrane filtration with subsequent washing of the cells, after which the radioactivity on the filter was counted. As a washing fluid 0.1 M Na-phosphate buffer (pH 6.8) was chosen in order to limit the losses due to washing of the cells.
Incubation of the preloaded cells was performed at 37^{o} under aerobic conditions to prevent growth. Loaded cells were resuspended in culture medium containing one of the growth inhibitors and in normal medium, and incubated simultaneously. The efflux of $^{86}Rb^{+}$ ions from inhibited cells was always compared with that from control cells of the same original batch. The results for a number of inhibitors are shown in Fig. 46. In the presence of inhibitors of protein biosynthesis (chloramphenicol, tetracyclin and actinomycin-D) the efflux

TABLE XVII. EFFECT OF GROWTH INHIBITION ON THE OSMOTIC BEHAVIOR OF PROTOPLASTS

The data in this Table are mean values from the indicated number of experiments. Details are given in Fig. 45.

Condition	Number of experiments	A_{550}-method			A_{260}-method		
		50% lysis experimental	50% lysis control	ΔL50	50% lysis experimental	50% lysis control	ΔL50
Chloramphenicol	4	0.34	0.11	+0.23	0.32	0.12	+0.20
Tetracyclin	3	0.23	0.12	+0.11	0.20	0.09	+0.11
Actinomycin-D	3	0.24	0.12	+0.12	0.24	0.10	+0.14
Penicillin G	4	0.14	0.27	−0.13	0.18	0.22	−0.04
Bacitracin	3	0.13	0.22	−0.09	0.12	0.23	−0.10
D-cycloserine	3	0.17	0.21	−0.04	0.20	0.26	−0.06
Vancomycin	3	0.11	0.21	−0.10	0.15	0.23	−0.08
Without milk (2.5 h)	2	0.32	0.25	−0.07	0.31	0.25	−0.06
Without milk (5 h)	2	0.28	0.32	−0.04	0.28	0.34	−0.06
Staphylococcin	2	0.13	0.16	−0.03	0.10	0.17	−0.07
Without Mg^{2+} (5 h)	3	0.50	0.33	+0.17	0.56	0.34	+0.22

of $^{86}Rb^+$ is significantly smaller than in normal cells. In the presence of inhibitors of peptidoglycan biosynthesis, as well as in the absence of human milk, an inverse effect is apparent, the $^{86}Rb^+$ efflux being increased. No effect was visible during inhibition by D-cycloserine. It was remarkable that the differences were already manifest immediately after incubation of the cells in the medium.

Thereafter the uptake of $^{86}Rb^+$ was determined in the presence and absence of growth inhibitors (Fig. 47). Cells cultivated for 10 h were resuspended in fresh medium (pH 6.8) containing $^{86}Rb^+$. In order to obtain a sufficiently large uptake of $^{86}Rb^+$, the K^+ concentration of the medium was lowered from 28 to 3 mM by replacing the K_2HPO_4 with Na_2HPO_4. Under these conditions we observed a detectable uptake of $^{86}Rb^+$ in the cells. The influence of inhibitors of protein biosynthesis (chloramphenicol, tetracyclin and actinomycin-D) was marked. Almost immediately after suspension of the cells in fresh, $^{86}Rb^+$-containing medium there was a larger uptake of $^{86}Rb^+$ in inhibited cells than in control cells. Inhibition of peptidoglycan synthesis by penicillin G, vancomycin, bacitracin, staphylococcin and depletion of human milk, caused an opposite effect. These cells showed an uptake in the early incubation time but an efflux occurred later. This effect was not significant in D-cycloserine-inhibited cells.

The experiments show roughly two effects of growth inhibition: a larger efflux and smaller uptake of $^{86}Rb^+$ during inhibition of peptidoglycan synthesis and a smaller leakage and larger uptake during inhibition of protein biosynthesis. The effects became manifest almost immediately after the cells were subjected to inhibitory conditions.

A second approach to obtain indications for release or uptake of electrolytes during growth inhibition was by studying the Na^+ and K^+ contents of the cells. Na^+ and K^+ are the major cations in the culture medium, which we also assumed to be present in considerable amounts intracellularly.

The cells were washed in a Na^+- and K^+-free medium, whereupon the Na^+ and K^+ contents were determined and expressed as µmoles per 1 culture per unit of absorbance. In testing the influence of growth

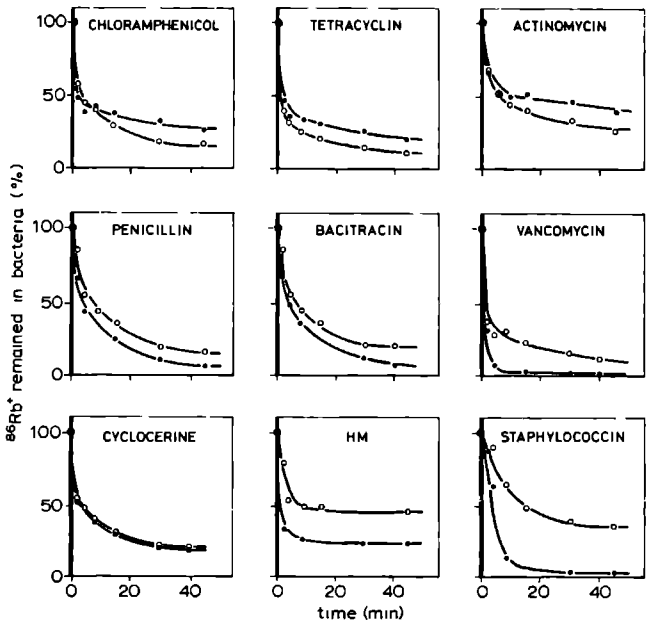

Fig. 46. Efflux of $^{86}Rb^{+}$ from normal and inhibited cells. The efflux was determined in the presence and absence of antibiotics and in medium from which human milk was omitted. For details of the procedure see *4.2.12.* (Open symbols: normal cells; solid symbols: inhibited cells.)

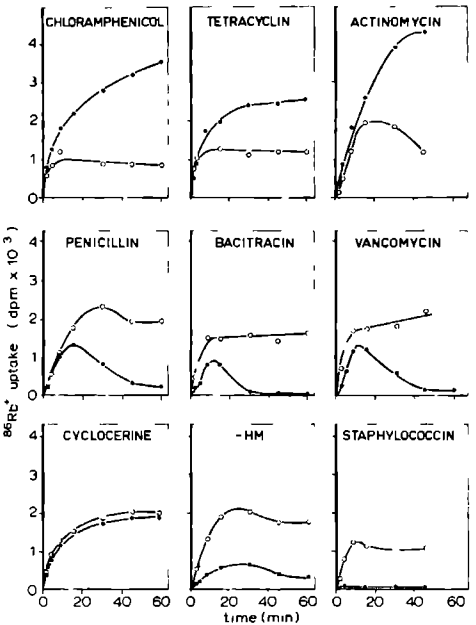

Fig. 47. Uptake of $^{86}Rb^{+}$ by normal and inhibited cells. Uptake was determined in medium with 3 mM K^{+} in the presence and absence of antibiotics and in medium from which human milk was omitted. For details of the procedure see *4.2.13.* (Open symbols: normal cells; solid symbols: inhibited cells.)

TABLE XVIII. EFFECT OF ANTIBIOTICS ON Na^+ AND K^+ CONTENTS OF CELLS OF B. BIFIDUM VAR. PENNSYLVANICUS

Cells from 10.5 h-cultures were incubated for 3 h in fresh medium with human milk and various antibiotics or without human milk. Contents are given as percentage of those of control cells and are means of 7 determinations.

	Concentration (µg/ml)	Na^+	K^+
Normal	-	100	100
Chloramphenicol	60	78	150
Tetracyclin	50	98	142
Actinomycin-D	10	43	100
Penicillin G	10	0	0
Bacitracin	160	131	17
Cycloserine	80	111	45
Vancomycin	20	131	0
Omission of human milk	-	104	81

inhibition on the intracellular Na^+ and K^+ contents we compared inhibited cells with control cells from the same original culture. The data are reported as percentages of the electrolyte content in normal cells (Table XVIII).

The intracellular Na^+/K^+ ratio in normal cells was about 0.8, which is far lower than that in the culture medium (about 11.6), indicating that the organism has the ability to extrude Na^+ ions and to accumulate K^+ ions. Inhibitors of protein synthesis generally caused a decrease in Na^+ content of the cells, but the K^+ content was increased significantly by chloramphenicol and tetracyclin. The K^+ content remained about the same during inhibition by actinomycin-D. A marked effect of inhibition of peptidoglycan synthesis was found for the K^+ content of the cells, which was decreased strongly by penicillin,

vancomycin, D-cycloserine, bacitracin and by depletion of human milk. The Na^+ content was increased by vancomycin, D-cycloserine and also slightly by depletion of human milk, but a strong decrease occurred during inhibition by penicillin.

4.4. DISCUSSION

4.4.1. Lipid-phosphorus, lipid-galactose, DNA and RNA content after inhibition of growth

In these experiments a period of active growth is involved since cells from the exponential growth phase (10 h) were incubated for 3 h in fresh culture medium at relatively low culture densities. During this period we found an increase of the lipid-phosphorus content of the cells, whereas the lipid-galactose content did not change significantly. This observation corresponds with other observations (Van Schaik and Veerkamp, 1975) that the lipid-phosphorus content of the cells appears to increase during the first hours of active growth. During inhibition of protein synthesis there was generally no increase in the lipid-phosphorus content. Van Schaik and Veerkamp (1975) found that inhibition of protein synthesis did not markedly affect the phospholipid content and composition.
Whereas the lipid-galactose content of the normal cells did not change significantly, it increased during inhibition by chloramphenicol and tetracyclin. Since the lipid-phosphorus content did not increase and the phospholipid composition was not markedly changed (Van Schaik and Veerkamp, 1975), this larger increase cannot be ascribed to an increased phosphogalactolipid content. It is probably due to an increase in the galactosyldiglycerides or to internal changes in their composition.
Influences of the inhibition of protein synthesis on the lipid-phosphorus content have been described in several reports. Chloramphenicol caused a general reduction of phospholipid synthesis in *E. coli* (Nunn and Tropp, 1972) and of lipid production of *B. amyloliquefaciens*. In the latter organism the effect was primarily on

phospholipids but not on glycolipids. There was no selective inhibition of any of the phospholipid components (Glenn and Gould, 1973). These observations agree with our results for the lipid-phosphorus content. Redai et al. (1973) found an altered phospholipid synthesis in *S. aureus* with chloramphenicol in concentrations that did not inhibit bacterial multiplication. The lysylphosphatidylglycerol content was reduced, the phosphatidylglycerol content was increased, but the diphosphatidylglycerol content was not affected. Ballesta and Schaechter (1971) reported that tetracyclin did not inhibit phospholipid synthesis and turnover in *E. coli*. In *Serratia marcescens*, low concentrations of streptomycin caused a doubling of the lipid-phosphorus content, which was mainly due to an increase of the phosphatidylethanolamine content (Bermingham et al., 1970). These results show that inhibition of protein synthesis does not necessarily imply that phospholipid synthesis is also decreased.

We showed that the incorporation of fatty acids continued during inhibition of protein synthesis (Chapter 2). This suggested that lipid synthesis and protein synthesis in the membrane are not necessarily coupled. The study of the lipid-phosphorus and lipid-galactose content during inhibition of protein synthesis also adds a new element: when protein synthesis is arrested, changes in membrane lipid composition may still occur.

During inhibition of cell wall synthesis the lipid-phosphorus content of the cells tended to increase more than in normal cells. This agrees with the results of Van Schaik and Veerkamp (1975), who found in ^{32}P-labeling experiments that during inhibition of peptidoglycan synthesis the lipid-phosphorus content was significantly higher than in normal cells. The increase was mainly due to an increase in the content of diphosphatidylglycerol and its lyso-derivatives. Bacitracin formed an exception, since it had little effect on the phospholipid composition (Van Schaik and Veerkamp, 1975).

The increase of the cardiolipin content in cells, which occurs especially during inhibition of cell wall synthesis in *B. bifidum* var. *pennsylvanicus*, is a phenomenon which has often been observed under various conditions in a variety of organisms as a consequence of either death or stasis of cells. It occurs in *S. aureus* in the

presence of relatively high concentrations of NaCl (Kanemasa et al., 1972); in *S. aureus* due to staphylococcin (Jetten, 1973); after glycerol deprivation in a *Bacillus subtilis* glycerol auxotroph (Lillich and White, 1971) and during phage infection (Petersen and Buller, 1969), action of colicins (Cavard et al., 1968) and in the presence of levorphanol (Wurster et al., 1971) and phenethyl alcohol (Nunn and Tropp, 1972) and on sodium inhibition (Lusk and Kennedy, 1972), in specific *E. coli* strains.

The influence of inhibition of cell wall synthesis on phospholipid metabolism was also studied by other investigators. Addition of penicillin or cephalosporin to growing cells of *Corynebacterium alkanolyticum* (Nakao et al., 1973; Kikuchi et al., 1973) caused simultaneous excretion of phospholipids, UDP-N-acetyl-hexosamine derivatives and L-glutamic acid. Other inhibitors of cell wall synthesis only promoted the excretion of UDP-N-acetyl-hexosamine derivatives. The excreted phospholipids had the same composition as the intracellular phospholipids, but in contrast to our organisms a significant decrease in phospholipid content of the cells appeared. In *Brevibacterium thiogenalis* (Kikuchi et al., 1973) penicillin triggered the excretion of L-glutamic acid and of phospholipids. However, in this case the phospholipid content did not decrease. Low concentrations of penicillin inhibited cell division in *E. coli*, causing filaments (Starka and Moravoa, 1970). Phospholipid synthesis was not altered but the ratio of individual lipids was changed. The filaments contained more cardiolipin and less phosphatidylglycerol than normal organisms. Glycine as an inhibitor of cell wall synthesis in *B. subtilis* caused a significant increase of diphosphatidylglycerol. There was probably no inhibition of phosphatidylglycerol synthesis (Sato et al., 1974). It is important to point to the fact that despite excretion of considerable amounts of phospholipids (Chapter 3) the lipid-phosphorus content of the cells did not decrease compared to that of normal cells. This implies that the excreted phospholipids are replaced by newly synthesized phospholipids or are the result of excess synthesis.
We have no information about the composition of the glycolipids after

inhibition of cell wall synthesis by antibiotics. However, data on the glycolipid composition of cells, cultivated in medium without human milk, showed a decrease in the lipid-galactose content. Compared with normal cells this decrease resulted both from a decrease of all galactosyldiglycerides and from a shift in the ratio of these components from digalactosyl to monogalactosyl lipids (Exterkate and Veerkamp, 1971) and from a decrease in the phosphogalactolipid content (Van Schaik and Veerkamp, 1975).
The lipid-galactose content in penicillin-inhibited cells was about the same as in control cells, although considerable amounts of glycolipids leave the cells of *B. bifidum* var. *pennsylvanicus* after addition of this antibiotic. This may be due to synthesis of new glycolipids or shifts in the ratio of individual components. A possible decrease of the lipid-galactose content by changes in the galactosyldiglycerides is not compensated by an increase in phosphogalactolipids, since the phosphogalactolipid content is lower than in normal cells (Van Schaik and Veerkamp, 1975).

Chloramphenicol blocks in intact cells protein synthesis, but DNA and RNA synthesis continues (Goldberg, 1965). Tetracyclin inhibits protein synthesis, but not the synthesis of DNA and RNA, which may be inhibited by actinomycin-D (Goldberg, 1965). Our results suggest that despite the absence of protein synthesis the synthesis of nucleic acids was still possible. It was also found that the DNA and RNA contents of the cells were not significantly smaller after 3 h incubation with antibiotics than at the start of the incubation indicating that no extensive leakage of intracellular materials occurred. The latter conclusion is also valid for antibiotics inhibiting peptidoglycan synthesis. These antibiotics appeared also to counteract the increase of DNA and/or RNA content rather strongly. This may indirectly cause the strong inhibition of the incorporation of [^{3}H]glycine into cellular protein (Chapter 2).

4.4.2. Changes in fatty acid composition during growth inhibition

The fatty acid composition of bacteria can be influenced by many

factors. These factors may comprise nutritional conditions, as shown for thermophilic Bacillus species (Daron, 1973); the growth temperature, as demonstrated for *E. coli* (Haest et al., 1969; Knivett and Cullen, 1965; Marr and Ingraham, 1962), *Serratia marcescens* Kates and Hagen, 1964) and *Micrococcus cryophilus* (Russel, 1971); and also the combined effect of pH and temperature in *B. acidocaldarius* (de Rosa et al., 1974). The growth phase of the cells may have an important influence on the bacterial fatty acid composition. During continuous ageing of the culture of *B. bifidum* var. *pennsylvanicus* an increase in the average chain length of the fatty acids was observed (Veerkamp, 1971).

Inhibition of protein synthesis in our experiments did not result in significant changes in the fatty acid composition of the membrane lipids. In Chapter 2 we showed that incorporation of oleic acid under conditions of arrested protein synthesis into the membrane fraction took place. The absence of changes in the fatty acid composition under these conditions suggests that it is not due to preferential incorporation of this fatty acid.

However, inhibition of peptidoglycan synthesis had a significant effect on the fatty acid composition. This effect involved an increase in octadecenoic acid with a concomitant decrease of octadecanoic acid in the presence of penicillin, vancomycin, a decrease of ocatadecanoic acid in the presence of D-cycloserine and bacitracin and a decrease of octadecanoic acid and octadecenoic acid in the presence of staphylococcin. The effects of the latter two inhibitors were relatively small. The results cannot be explained by assuming an accelerated ageing process of the cells.

We have no reports about the influence of antibiotics inhibiting cell wall synthesis on the fatty acid composition of bacterial lipids. However, for a number of bacterial organisms the fatty acid composition depends on the presence of a cell wall. L-forms (organisms lacking a cell wall) of *S. pyogenes* contain relative more 18:1 and less 16:0 fatty acids than the parent strain with a normal cell wall. This is not a general phenomen, as appears with the L-forms of *S. aureus*, which show no important differences in fatty acid composition relative to the parent organism (Ward and Perkins, 1968).

L-forms of Proteus P-18 showed a considerable decrease in C_{17}- and C_{19}-cyclo fatty acids (Nesbitt and Lennarz, 1965). Cells of *B. bifidum* var. *pennsylvanicus*, after cultivation in medium lacking human milk, showed a replacement of octadecanoic acid by hexadecanoic and octadecenoic acid with a resulting decrease in the effective chain length. This effect could be attributed largely to a difference in growth phase (Veerkamp, 1971).

The increased incorporation of oleic acid during inhibition of peptidoglycan synthesis may be influenced in some cases by changes in the fatty acid patterns of the lipids. This is, however, not the main reason for the increase in incorporation, shown by the following observations: (i) increased oleic acid incorporation was also observed with bacitracin and staphylococcin (decrease of octadecenoic acid); (ii) the incorporation of palmitic acid during inhibition of peptidoglycan synthesis increased, whereas the hexadecanoic acid decreased (penicillin, vancomycin, D-cycloserine).

The increase in octadecenoic acid in Mg^{2+}- and sodium acetate-depleted cells seems characteristic for an ageing process, but the decrease of the octadecanoic acid is not compatible with this idea. It may be possible that the observed incorporation of oleic acid (Chapter 2) in cells depleted from sodium acetate can at least in part be ascribed to changes in the fatty acid composition. This may also be the case for the incorporation in Mg^{2+}-depleted cells (Chapter 2).

4.4.3. Composition of membrane protein

The amino acid composition of membrane proteins was not changed significantly by antibiotics or Mg^{2+}-depletion of the culture medium. The results obtained for inhibition of peptidoglycan synthesis agree with those for the membrane protein composition of *S. aureus* and its L-forms. There were only slight differences with the relative amounts of amino acids in their membrane preparations (Ward and Perkins, 1968). The same is true for membrane preparations of *S. pyogenes* and its stabilized L-form (Panos et al., 1972) and for the membrane of *B. bifidum* var. *pennsylvanicus* cultivated with and without human milk for 16 h (Exterkate et al., 1970).

Theoretically the membrane protein composition might alter without affecting the overall amino acid composition significantly. In the membrane preparation of normal and inhibited cells of *B. bifidum* var. *pennsylvanicus* no qualitative differences could be detected in protein composition. It is still possible that there are qualitative changes which were too small to be detected or which coicided in the electropherograms with other protein bands. The quantitative amounts of different membrane proteins were not determined. Quantitative changes may occur in the protein composition without detectable changes in the electropherograms or in the amino acid composition of the membrane. This has been demonstrated for *S. pyogenes* and its stabilized L-form (Panos et al., 1972). Changes in the membrane protein composition of *E. coli* cells occurred upon inhibition of cell division. There was a good correlation between the increase of a certain protein (protein X) and inhibition of cell division by mutation, nutritional conditions and inhibitors (Inouye and Pardee, 1970). To detect such changes in protein composition in our experiments a more sensitive version of the technique would have to be used.

4.4.4. Osmotic behavior of protoplasts from normal and inhibited cells

Within moderate ranges of osmotic external pressure protoplasts behave as perfect osmometers (Gilby and Few, 1959; Marquis, 1967) and follow the Lucké and McCutcheon modification of the Van 't Hoff-Boyle equation: $P(V - Vo) = k$, where P = osmotic pressure of the surrounding medium, V = total cell volume, Vo = osmotically inactive volume and k is a constant (Lucké and McCutcheon, 1932). The response of protoplasts to osmotic forces is determined by the interactions of three components: internal phase, external phase and the interacting membrane barrier (Eisenberg and Corner, 1973). The interacting membrane barrier influences the osmotic response by its mechanical properties but also by its permeability. This permeability may regulate the internal osmotic pressure and may also determine the admission of water and large molecules, a process preceeding lysis of protoplasts in hypotonic media. In hypotonic media protoplasts will

swell according to the Lucké and McCutcheon formula. However, as we have indicated the swelling is not unlimited. The process of swelling is regulated by the limiting membrane of the protoplast. Marquis (1965) found indications that the protoplast is perforated by small aqueous channels of variable diameter. During swelling these aqueous channels effectively dilate and can admit large molecules (Marquis and Corner, 1967). If the membrane stretches enough to permit stabilizing solute molecules to enter the protoplasts, there is a rapid influx of solutes and water resulting in a rapid stretching of the membrane. Bursting of the protoplasts will occur due to brittle fracture of the membrane (Corner and Marquis, 1969). Brittle fracture is determined to a great extent by the mechanical properties of the membrane. Corner and Marquis (1969) indicated that the mechanically most important membrane component is the protein.

We found that inhibition of protein biosynthesis and omission of Mg^{2+} led to an osmotic behavior in which the protoplasts tended to burst at a lower osmotic gradient than control protoplasts. The relatively increased instability of the protoplasts after Mg^{2+}-omission was expected, since Mg^{2+} is needed for the structural integrity of the cytoplasmic membrane. However, the effect of inhibition of protein biosynthesis could be due to changes in the structural integrity of the membrane, in the mechanical properties of the membrane or in the internal osmotic pressure (which may be dependent on the membrane permeability).

The relatively increased stability of the protoplasts after inhibition of cell wall synthesis may be due to changes in the same parameters. We have shown in preceding sections that during inhibition of protein biosynthesis and cell wall synthesis changes in the lipid composition occur. This might affect the protoplast behavior. We cannot give a clear answer to the question whether the differences in osmotic behavior of the protoplast of inhibited cells must be explained on the basis of altered composition and/or mechanical properties of the membranes or that these were due to altered permeability and subsequent changes in the internal osmotic pressure. The latter changes might take place during inhibition of the cells, during protoplast formation or during subjection of the protoplasts

to hypotonic media. We performed a number of experiments which showed a correlation between the changes in osmotic properties of protoplasts and changes in permeability of the membrane with concomitant losses or gains in osmotically active material during inhibition of intact cells.
It is interesting to note the experiments of Exterkate et al. (1970), comparing the osmotic properties of protoplasts of normal cells of *B. bifidum* var. *pennsylvanicus* and of cells cultivated in the absence of human milk. They detected two fractions of different stability, one fraction being more labile and one fraction being more stable than protoplasts from normal cells. They indicated the possibility that the apparent mechanical stability in hypotonic solution of the protoplasts of inhibited cells can be explained by the release of osmotically active material from the protoplasts, resulting in an increase in cytoplasmic water activity.

4.4.5. Efflux and uptake of electrolytes

Significant changes in the uptake and loss of electrolytes from cells have been demonstrated by action of inhibitors of protein biosynthesis and of cell wall biosynthesis. The former group of inhibitors (except for actinomycin) stimulated uptake of $^{86}Rb^{+}$ and increased the cellular K^{+} content, whereas the latter group caused an increased efflux of $^{86}Rb^{+}$ from cells and decreased their K^{+} content.
The changes in osmotic behavior of the protoplasts after inhibition of the cells (except for actinomycin-D) can be explained by the lower content of electrolytes in the cells after inhibition of peptidoglycan synthesis and the higher content after inhibition of protein biosynthesis (Table XIX). These changes in electrolyte contents were predominantly determined by movements of K^{+} ions, although the Na^{+} ions also played a role. Changes in the electrolyte content might cause differences in the protoplast volume. It has been shown for swollen Ehrlich ascites tumor cells that these cells can partly regulate their volume by decreasing the amount of intracellular K^{+}. The intracellular Na^{+} concentration remained unchanged. The passive membrane permeability was unaffected (Hendil

TABLE XIX. CORRELATIONS BETWEEN GROWTH INHIBITION, PROTOPLAST STABILITY AND LOSS AND UPTAKE OF ELECTROLYTES

Condition	Protoplast stability	Uptake $^{86}Rb^+$	Loss $^{86}Rb^+$	K^+ content	$Na^+ + K^+$ content
Chloramphenicol	<	>	<	>	>
Tetracyclin	<	>	<	>	>
Actinomycin-D	<	>	<	=	<
Penicillin G	>	<	>	<	<
Vancomycin	>	<	>	<	<
D-cycloserine	>	<	>	<	<
Bacitracin	>	<	>	<	<
Without milk	>	<	>	<	<
Staphylococcin	>	<	>	N.D.	N.D.

Meaning of the notation:

> : larger than in normal cells

< : smaller than in normal cells

= : about the same as in normal cells

N.D.: not determined

and Hoffmann, 1974).

Suspensions of cells of a marine pseudomonad in a K^+-depleted medium showed an immediate increase in absorbance when the NaCl concentration of the suspending medium was increased. In the presence of K^+ a subsequent slow decrease in absorbance occurred. The rate of this latter change was similar to the rate of uptake of ^{42}K by cells (Matula et al., 1970). It was concluded that the K^+-dependent decrease in absorbance was due to increase in cellular volume by K^+-accumulation. Protoplasts from *S. faecalis*, stabilized in 0.4 M sucrose, undergo a remarkable swelling when metabolizing small amounts of glucose, provided that K^+ or certain other cations are present (Abrams, 1959). The swelling of mitochondria with associated K^+ uptake is also an osmotic phenomen (Agata and Rasmussen, 1966).

It has been indicated (Marquis, 1965) that in protoplasts with a relatively large volume the aqueous channels might be more dilated than in normal protoplasts. In these cells only a relatively slight stretching of the membrane (caused by a decrease of external osmotic pressure) is sufficient to admit the stabilizing solute molecules and water with subsequent brittle fracture and lysis. Thus the intracellular cation concentration could have important consequences for the osmotic behavior of the protoplasts. This has been shown for L-forms of *S. faecalis* (Montgomerie et al., 1972). An L-form of *S. faecalis* (T_{53}) was adapted to grow in medium without an osmotic stabilizer (sucrose). This adapted L-form had a relatively lower intracellular content of Na^+ and K^+ and a higher osmotic stability. However, we cannot explain the different behavior of actinomycin-D-inhibited protoplasts. The changes in electrolyte content cannot explain the osmotic behavior in this case. Possibly, actinomycin-D has also a more general effect on the mechanical stability of the membrane.

The cells of *B. bifidum* var. *pennsylvanicus* appear to possess a regulatory mechanism for their intracellular Na^+ and K^+ content. Veerkamp (1975, to be published) showed that the cells of *B. bifidum* var. *pennsylvanicus* accumulate K^+ from the medium and extrude Na^+ from the cells, which explains the difference in the Na^+/K^+ ratios of cells and culture medium. Such regulatory mechanisms have also been reported for other organisms (*E. coli*: Schultz and Solomon, 1961; Schultz et al., 1962; Schultz et al., 1963; Hafkenscheid and Bonting, 1971; *S. faecalis*: Zarlengo and Schultz, 1966; Harold et al., 1969; Harold et al., 1970; Abrams and Smith, 1971).

Few reports about the influence of antibiotics on the intracellular Na^+ and K^+ contents are known. The results of these investigations are, however, in line with our observations. At concentrations inhibitory to peptidoglycan synthesis, penicillin, bacitracin and vancomycin caused an increased efflux of K^+ ions from growing bacteria of *B. megaterium* (Hancock and Fitz-James, 1964). This effect was prevented by chloramphenicol or hypotonic sucrose solutions for penicillin, but not for vancomycin and bacitracin. The prevention of K^+ efflux with chloramphenicol might in our opinion represent

an antagonizing effect through inhibition of protein biosynthesis, as we have found in our experiments. The influence of the hypotonic sucrose solution indicates the role of the external osmotic pressure of the medium. Cells of *S. faecalis*, incubated in osmotically stabilized medium, accumulated rather than extruded Na^+, but accumulated less K^+ in the presence of penicillin. There was no effect of penicillin in non-growing cells and in stable protoplasts (Montgomerie et al., 1968). This shows that the influence of penicillin on the K^+ accumulation is probably not caused by a direct action of penicillin on the membrane. Montgomerie et al. (1968) concluded that these effects are related to the production of penicillin-induced lesions in the cell wall.

Our results suggest that the decrease of K^+ content and the increased efflux of $^{86}Rb^+$ are a more general effect of inhibition of cell wall synthesis. This effect might be on an existing K^+-H^+ exchange system, an Na^+-K^+ transport system or a change in membrane permeability. The latter possibility is most probable, since Van Schaik and Veerkamp (1975) and we also showed changes in the lipid composition of the membranes. There are several indications that in microorganisms the membrane permeability is influenced by the chemical composition of the membrane. Haest et al (1972) showed that non-electrolyte permeability and permeability for Rb^+ ions in liposomes and in intact cells of *S. aureus* depended strongly on the phospholipid composition. Op den Kamp et al. (1967) showed that protoplasts of *B. megaterium*, harvested from different growth phases, had a different membrane lipid composition and displayed also a different behavior during lysis in hypotonic sucrose solution. Op den Kamp et al. (1972) showed that degradation of phospholipids in the protoplast membrane of *B. subtilis* with phospholipase A_2 and C resulted in lysis in hypotonic media. These studies did not take into account that changes in the internal phase of the cells might have taken place due to changes in lipid composition.

Eisen berg and Corner (1973) induced changes in the lipid fatty acid composition of membranes of *B. megaterium* by growth at different temperatures. Protoplasts from cells grown at high temparatures contained relatively more saturated fatty acids and were more stable

tahn from cells grown at low temperatures.

Membrane permeability is possibly correlated to the fatty acid composition of the membrane lipids. In liposomes of synthetic lecithins non-electrolyte permeability was enhanced by introduction of fatty acids with double bonds or short chain length into the phospholipid (de Gier et al., 1968). Scarpa et al. (1971) showed an increasing permeability for K^+ ions with increasing unsaturation of the membrane fatty acids. There was a discrimination between Na^+ and K^+ permeability since the Na^+ permeability was increased much less than that for K^+ ions. Intact cells of *Acholeplasma laidlawii*, as well as liposomes derived from the lipids of these cells, showed an increased non-electrolyte permeability when branched chain, unsaturated or short chain fatty acids were incorporated into the membrane lipids (McElhaney et al., 1973). De Kruyff et al., (1973) demonstrated that the equilibrium flux of erythritol in *Acholeplasma laidlawii* was dependent on the fatty acids present in the membrane of the organism. The equilibrium flux became lower when the unsaturation of the fatty acids on which the organism was grown, was decreased. Van der Neut-Kok et al. (1974) reported that the valinomycin-induced permeability of *Acholeplasma laidlawii* for Rb^+ and K^+ increased with increasing unsaturation of the membrane lipids. Efflux of K^+ decreased at lower temperatures and became zero below the temperature at which the gel-liquid crystalline phase transition of the membrane lipids took place. The authors suggested that membrane fluidity is involved in membrane permeability.

We have shown that marked changes in the fatty acid composition of the predominant membrane lipids occur during inhibition of peptidoglycan synthesis. The changes in fatty acid composition may have affected the physical properties of the membrane, which are related with the membrane permeability. For most, but not all, inhibitors of peptidoglycan synthesis we observed an increased unsaturation of the fatty acids of the membrane lipids. Such an effect may increase the membrane permeability for K^+ and Rb^+ ions and explain the observed efflux for these ions. However, we cannot explain the effects of the inhibition of protein synthesis in this way, since we did not find

significant effects on the fatty acid composition. Not only effects on the membrane fatty acid composition were observed, but also marked changes in the galactolipid and phospholipid content. The lipid-phosphorus content was markedly increased and the phospholipid composition was changed after inhibition of peptidoglycan synthesis, whereas during inhibition of protein synthesis the lipid-galactose content was markedly increased. These changes in membrane lipid composition might have an important effect on the membrane permeability.

CHAPTER 5

ELECTRON MICROSCOPIC STUDY OF THE INFLUENCE OF ANTIBIOTICS ON THE CELLULAR ULTRASTRUCTURE

5.1. INTRODUCTION

In the preceding chapter we have described the effects of antibiotics and nutrient depletion on the cell envelope of *B. bifidum* var. *pennsylvanicus*. An electron microscopic study was made to obtain additional information about changes in utrastructure of the organism which might possibly explain the biochemical changes observed. The major points of interest were cell destruction and alterations in the cell wall and cell membrane after inhibition of cell wall and protein biosynthesis.

5.2. METHODS

5.2.1. Cultivation of the cells

Cells were cultivated for 10 h at 37° and resuspended in fresh culture medium (pH 6.8) with and without antibiotics. The concentrations of the antibiotics are given in 2.2. For the study of the effects of deprivation of N-benzoyl-glucosamine, cells were incubated in a culture medium (pH 6.8) without N-benzoyl-glucosamine. The cells were incubated for 3 h at 37° under N_2/CO_2 (95:5, by vol).

5.2.2. Fixation, staining and electron microscopy

Cells were prefixed during 10 min with glutaraldehyde (added to the culture to a concentration of 5%). The culture was centrifuged and the pellet embedded in 2% Difco Bacto-agar. The agar was cut into 1 mm^3 cubes. Subsequently the cells were fixed overnight at room temperature with 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), washed during 2 h in 0.1 M veronalacetate buffer (pH 6.0)

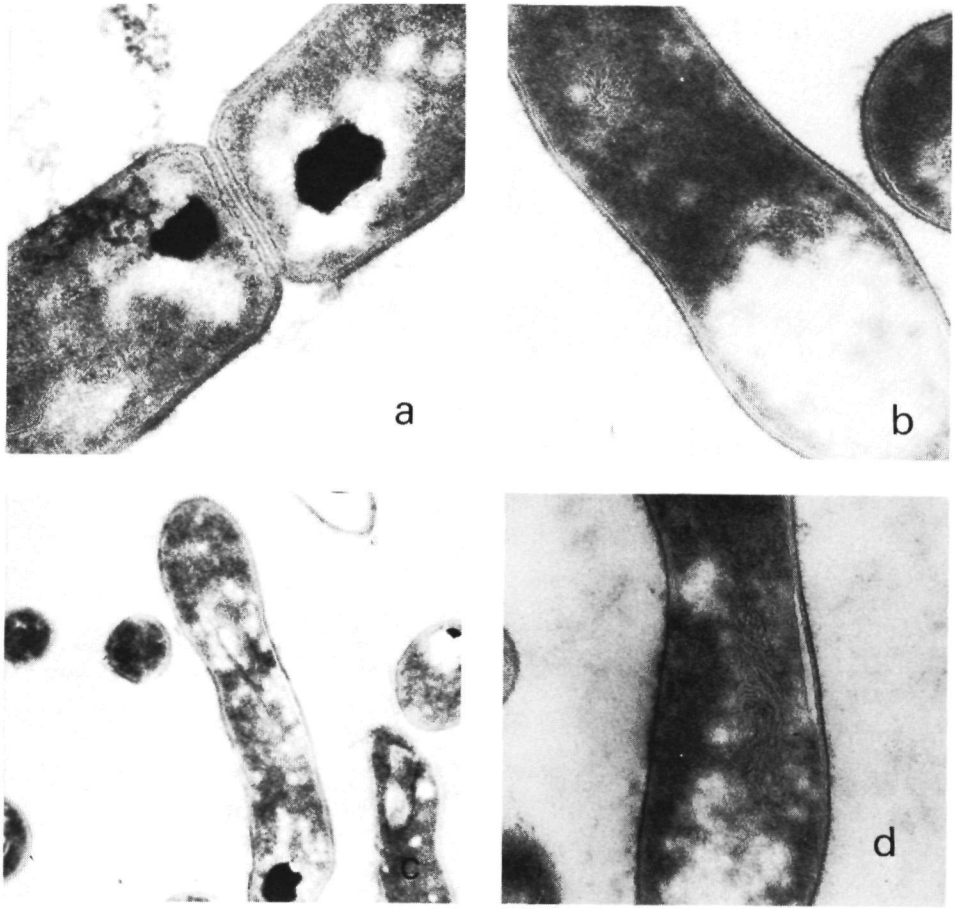

Fig. 48. Electron micrographs of cells of *B. bifidum* var. *pennsylvanicus*. a. Normal cells showing cell wall and plasma membrane and black bodies. b and d. Cells after a 3 h incubation period with tetracyclin showing the occurrence of electron-transparent gaps in the plasma membrane. c. Cells after a 3 h incubation period with chloramphenicol also showing electron-transparent gaps. Magnification: a: 74 000 x; b: 77 000 x; c: 22 000 x; d: 32 500 x.

and post-fixed overnight in 1% OsO_4 in 0.1 M veronalacetate buffer (pH 6.0) (Ryter and Jacob, 1966). Block-staining was performed with 0.5% uranylacetate in veronalacetate buffer during 2 h at room temperature. The cells were then dehydrated in graded ethanol solutions and embedded in Epon 812 (Luft, 1961). Post-staining in thin sections was performed with a half-saturated uranyl acetate solution (20 min) and with a leadcitrate solution according to Reynolds (1963) (10 min). The preparations were examined with a Philips EM 200 or with a Philips EM 300 electron microscope, equipped with a goniometer.

5.3. RESULTS

5.3.1. Normal cells

Electron microscopy of fixed and stained organisms from the normal 10 h cultures revealed the presence of rod-shaped cells with lengths in the order of 2.5 μ. Some of the cells are dividing (Fig. 48a). The bacterial cell wall is visible as an electron-dense layer at the outside of the cell. In intimate contact with this cell wall is the triple layered plasma membrane which is characteristic for Gram-positive organisms (Van Iterson, 1965). To the surface of the cells some diffuse material is attached which might represent carbohydrate material present at the outside of the cell wall. The content of the cells has a granular appearance in which some thready-like structures may occur. This material might be of nucleoid origin.

A number of cells contain dark bodies, the nature of which is unknown. Such bodies have also been observed in electron microscopic studies of *B. bifidum* by Overman and Pine (1963) and Poupard et al. (1973). Overman and Pine (1963) suggested that these bodies are bacteriophages. However, this is not very probable, in view of the dimensions of the dark bodies. In *Pseudomonas aeruginosa* coarse, electron-dense granules associated with the nucleoplasm were reported (Lickfeld, 1965). These granules resemble the particles in *B. bifidum* var. *pennsylvanicus*. According to Harold (1966) such granules are considered diagnostic

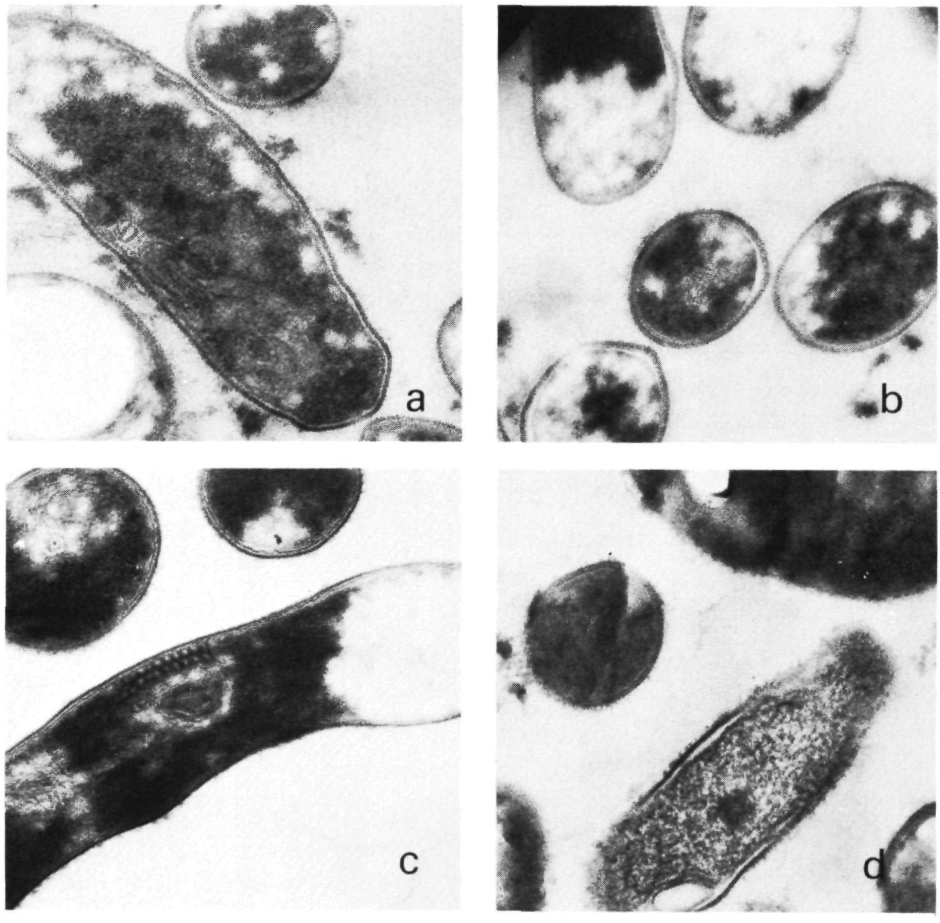

Fig. 49. Electron micrographs of cells of *B. bifidum* var. *pennsylvanicus* after a 3 h incubation period with actinomycin-D, showing the occurrence of electron-transparent gaps in the plasma membrane (a and b). The occurrence of mesosomal structures in cells inhibited by chloramphenicol is illustrated in (c); plasmolysis of normal cells after a 1 h incubation period in 0.3 M sucrose in (d).

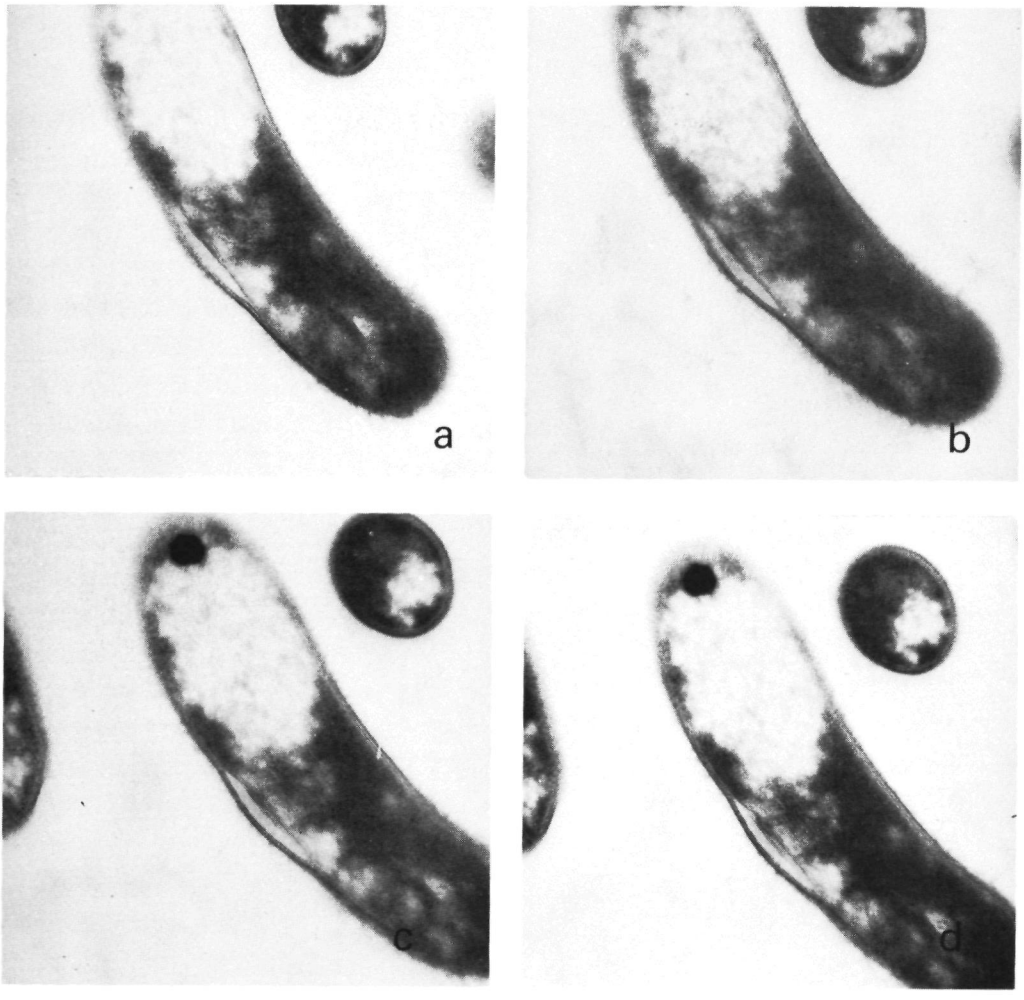

Fig. 50. Electron micrographs of cells of *B. bifidum* var. *pennsylvanicus* after a 3 h incubation period with tetracyclin. In respect of the perpendicularity of the electron beam the specimen was tilted over a 14^{o} angle (a), a+4^{o} (b), a-12^{o} (c) and a-21^{o} (d). These micrographs clearly demonstrate the existence of the two electron-dense leaflets of the plasma membrane on either side of the electron-transparent gap. Magnification: 100 000 x.

of polyphosphate.
In a few cells invaginations of membranes, membranous whorls or vesicles occurred. These structures are probably "mesosomes" which are often found in Gram-positive organisms. For a discussion about the reality of mesosomal structures we refer to *1.2.5.*

5.2.3. Inhibition of protein biosynthesis

Chloramphenicol, tetracyclin and actinomycin-D, inhibitors of protein synthesis have identical effects on the cellular ultrastructure (Fig. 48.b-d, Fig. 49.a-c). In general the inhibited cells showed a normal morphology. The cell wall showed no irregularities or abnormal thickness. Under the cell wall the unit-membrane structure was present. However, in some cells gaps between the cell wall and the outer dense leaflet of the plasma membrane were present. Occasionally this latter effect was also found in some normal cells after they were incubated for 1 h in a 0.3 M sucrose-containing medium (Fig. 49d). This effect was very suggestive of plasmolysis. In a significant number of cells the two electron-dense layers of the unit-membrane were separated by an electron-light gap which was considerably wider than in normal cells. The widening of this intermediate layer varied considerably among the cells and within one cell the phenomenon occurred only at a few places. In normal cells gaps between the electron-dense leaflets of the plasma membrane or between the cell wall and the plasma membrane did not occur. The existence of a true electron transparent gap between the two electron-dense leaflets of the unit-membrane was clearly demonstrated by examination of the (fixed and stained) preparations using a goniometer (Fig. 50.a-d). As can be seen a clear observation of this phenomenon is dependent on the angle between the perpendicularity of the electron beam and the preparation. The existence of an electron-dense leaflet on either side of the gap which originated from the plasma membrane was established.
In a great number of inhibited cells mesosomal structures were present (Fig. 49.c). Although these structures are found in normal cells they were more frequently observed in inhibited cells.

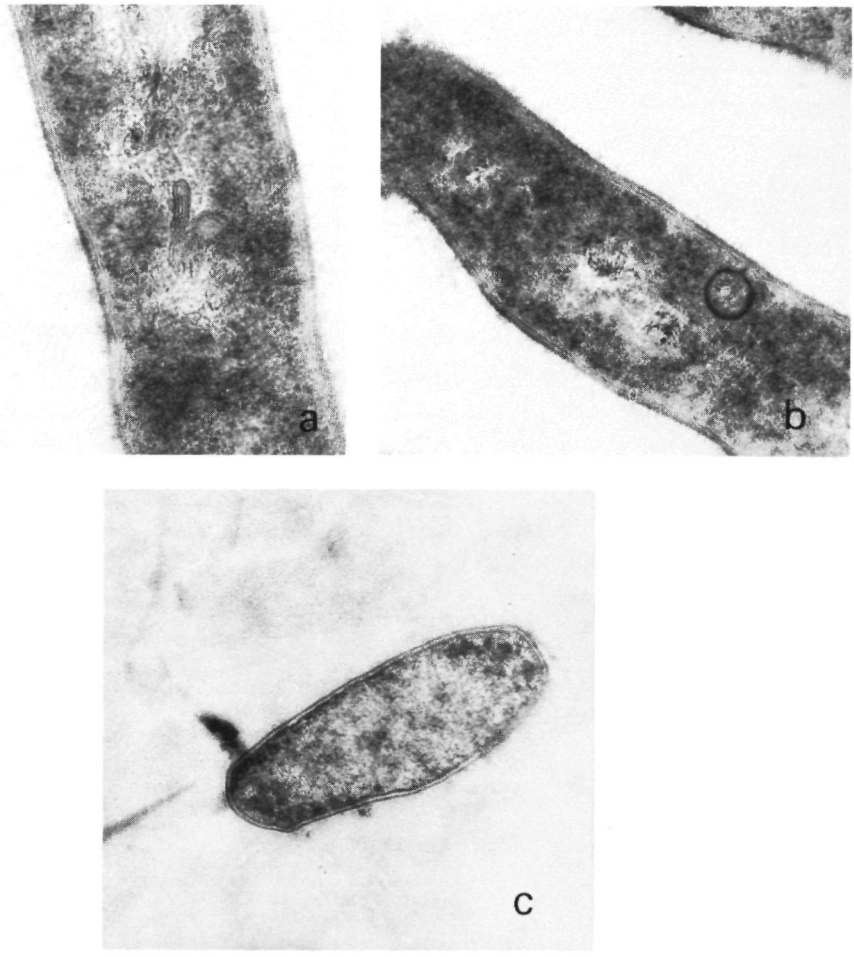

Fig. 51. Electron micrographs of *B. bifidum* var. *pennsylvanicus*. Cells after 3 h incubation with penicillin (a) and vancomycin (b), showing the occurrence of mesosomal structures. Cells after 16 h growth in a 0.14 mM Mg^{2+} medium with an electron-lucent gap between the cell wall and the plasma membrane (c).
Magnification: a: 62 000 x; b: 55 700 x; c: 28 400 x.

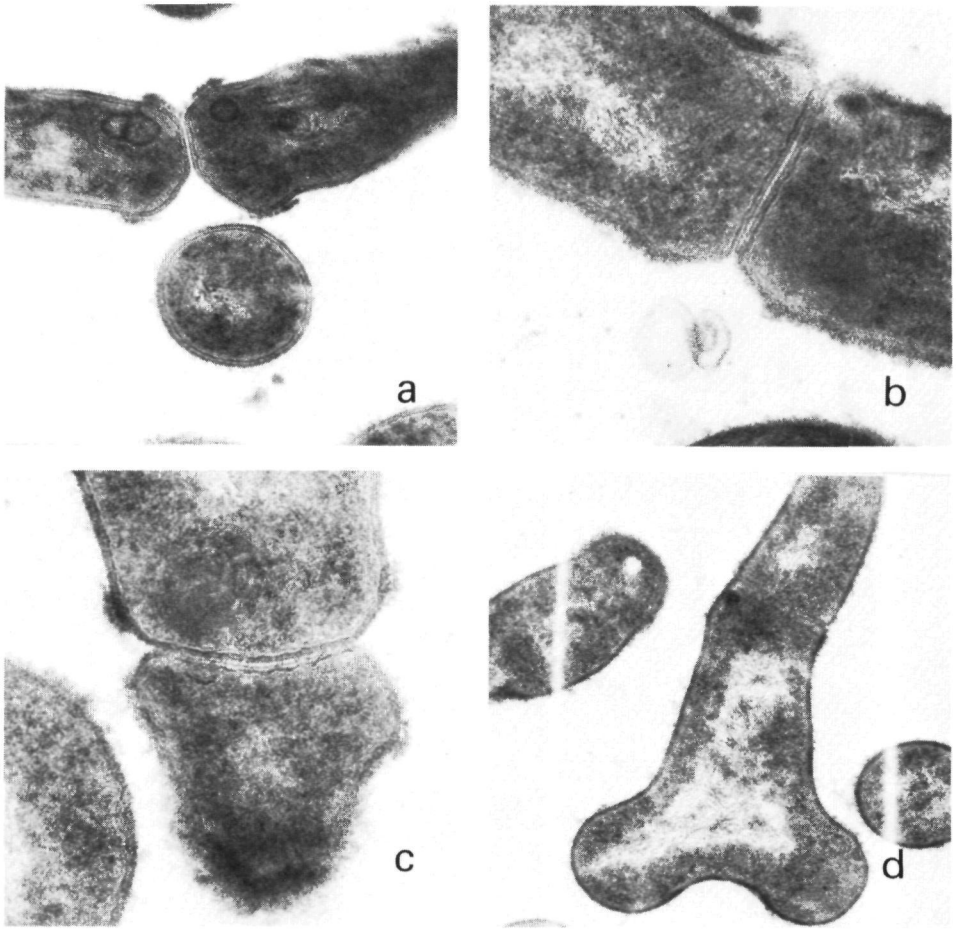

Fig. 52. Electron micrographs of *B. bifidum* var. *pennsylvanicus* after 3 h incubation in medium without N-benzoyl-glucosamine (a-c) with rings of electron-dense material and cells after 16 h incubation in medium lacking N-benzoyl-glucosamine showing bifurcation (d). Magnification: a and d: 39 200 x; b and c: 85 600 x.

5.3.3. Inhibition of peptidoglycan biosynthesis

When cells were incubated for 3 h in the presence of penicillin, vancomycin or bacitracin, there were no marked changes in morphology and ultrastructure (Fig. 51.a-b) We did not observe structural defects in the cell wall or in the plasma membrane. Only a few lysed cells were observed. The cell wall and plasma membrane always appeared to be in close contact and the cell membrane retained its characteristic unit membrane structure. The number of mesosomal structures observed in inhibited cells was increased.

Inhibition of peptidoglycan synthesis by deprivation of N-benzoyl-glucosamine during 3 h generally showed no large alterations in the cell morphology nor in the structure of the cell wall and the plasma membrane (Fig. 52.a-c). However, in a significant number of cells the occurrence of a ring of electron-dense material on the bacterial surface was remarkable. This electron-dense material was always deposited at one of the polar sites of the cell. In dividing cells such rings are always found symmetrically to the plane of division. It seems likely that the material originates from the region of the septum. After 16 h of incubation in a N-benzoyl-glucosamine-lacking medium such rings of electron-dense material were not observed. It is noteworthy that the morphology of the cells under these conditions had drastically changed into the characteristic bifid appearance (Fig. 52.d).

5.3.4. Inhibition of growth by omission of magnesium ions

For the study of the effect of Mg^{2+}-omission we used the normal cultivation time of 16 h. After omission of Mg^{2+}, cells had a normal electron microscopic morphology. In the electron microscopic study we found no significant alterations in cell wall and plasma membrane. The plasma membrane exhibited the characteristic unit-membrane structure. However, in a great number of cells a relatively broad electron-lucent gap between cell wall and cell membrane was present (Fig. 51.c).

5.4.1. Inhibition of protein biosynthesis

In the preceding investigations we showed that after arresting protein synthesis in *Bifidobacterium bifidum* var. *pennsylvanicus* the synthesis of peptidoglycan and lipids was not arrested. A continued peptidoglycan synthesis in cells of which the growth is inhibited will cause an relative overproduction of peptidoglycan per cell. We could show that peptidoglycan is not lost from the cells (3.3). The surplus peptidoglycan therefore must be stored in the cell or at the cell surface. In many Gram-positive organisms indeed cell wall thickening occurs after inhibition of protein biosynthesis: *B. cereus* (Chung, 1971; Chung, 1973), *B. subtilis* (Miller et al., 1967; Hughes et al., 1970; Frehel et al., 1971), *B. megaterium* (Frehel et al., 1971), *S. faecalis* (Higgins and Shockman, 1970) and *S. aureus* (Hash and Davis, 1962; Giesbrecht and Ruska, 1968; Nakao et al., 1972). These effects were obtained either by action of antibiotics or by amino acid starvation. The thickening of cell wall was observed after relatively short periods (1-4 h). In *B. bifidum* var. *pennsylvanicus* we could not establish an increase in cell wall thickness after inhibition of protein biosynthesis. Theoretically there might be several explanations: (i) the incubation period was too short to obtain a clear effect; (ii) the effects are too small to be detected; (iii) the deposit of surplus peptidoglycan causes no cell wall thickening.

The length of the incubation period, after which the effects on the cell wall were studied (3 h), appeared us long enough compared to the incubation times after which the effects in other organisms were observable (1-4 h). However, a factor of importance is whether an effect is easily detectable. The electron micrographs of *B. bifidum* var. *pennsylvanicus* show a relatively thin cell wall. Small increases of the cell wall thickness are in such walls not easily observable and require the application of accurate determination methods. This might explain the fact that we could not settle a thickening of the cell wall.

In our electron micrographs of inhibited cells we found a number of

cells (estimated, 1-2%), in which the cell wall and plasma membrane were at some places separated by an electron-transparent gap. A remarkable agreement was found with cells cultivated in Mg^{2+}-poor medium. In normal cells such electron-lucent gaps between cell wall and plasma membrane were never observed, but they could be produced in some cells by inducing plasmolysis with hypertonic sucrose media. We must bear in mind the possibility that the electron-lucent gaps between cell wall and plasma membrane are artifacts of the histological processing of the cells. In electron micrographs of fixed and sectioned cells of Gram-negative organisms a transparent zone between the plasma membrane and the peptidoglycan layers often occurs (Costerton, 1970; Murray et al., 1965; de Petris, 1967). However, there is no proof that this space exists in living cells (Costerton et al., 1974). An argument that the electron-lucent zone between cell wall and plasma membrane would not occur, is that the turgor pressure of the cell would force the plasma membrane layer outward against the relatively ineleastic peptidoglycan sacculus. It is known from plant cells that the relation between plasmalemma and the cell wall may be drastically altered by fixation (Costerton, 1970). The relation between cell wall and plasma membrane of *B. bifidum* var. *pennsylvanicus* might be disturbed in a similar way by fixation. If the electron-dense zones between cell wall and cell membrane are artifacts of fixation then they might reflect an alteration in the interaction between membrane and cell wall compared with normal cells.

In a great number of cells (estimated 15-25%) the distance between the electron-dense layers of the unit membrane was increased considerably after inhibition of protein biosynthesis. In *S. faecalis* it was found that starvation of essential amino acids (threonine or valine) appeared to alter the structure of both mesosomal membranes and cytoplasmic membranes by increasing the distance between the outer and inner electron-dense layers of the unit membrane (Higgins and Shockman, 1970). Higgins and Daneo-Moore (1972) found that all treatments causing inhibition of protein biosynthesis made the middle transparent layer of the protoplast membrane increase in thickness. This increase was attributed to an increase in the lipid

to protein ratio or to a change in the composition of the membrane lipids (Shockman et al., 1974). We established that during the inhibition of protein synthesis the synthesis of lipids in *B. bifidum* var. *pennsylvanicus* continued and that there were changes in the composition of the membrane lipids. This would imply an increased lipid content of the membranes concomitant with changes in the lipid composition. These changes could be responsible for the increased distance between the electron-dense layers of the membrane in *B. bifidum* var. *pennsylvanicus*.

A clear effect of inhibition of protein synthesis was the more frequent occurrence of nesosomal structures. Since mesosomes are known to possess lipids (*1.2.5*), these mesosomes might function as deposits for the lipids prduced in excess. These lipids have to be deposited in the cells since we established that no lipids leak from the cells during inhibition of protein synthesis. In *S. faecalis* the average amount of mesosomal membrane was increased upon chloramphenicol action (Higgins and Daneo-Moore, 1972). This was also during the first hours of amino acid starvation (Higgins and Shockman, 1970) in the same organism.

5.4.2. Inhibition of peptidoglycan synthesis

a. Action of antibiotics

Antibiotics which inhibit cell wall synthesis did not cause significant alterations in the cellular ultrastructure and morphology. There were no extensive signs of cellular destruction. This observation is in agreement with the finding (3.3) that almost no cellular protein leaves the cells after inhibition of peptidoglycan synthesis. The cell wall and plasma membrane showed no defects and were always in close contact with each other. Although we could not observe distinctive changes in the structure of cell wall and plasma membrane and in the cellular morphology, such changes have been reported for other organisms. D-cycloserine caused cells of *S. aureus Wood* and *S. pyogenes* to have thinner cell walls than normal cells and *S. zymogenes* to occur in elongated forms consisting of two to four

associated cells (Bisaillon et al., 1973). A thinner peptidoglycan layer was also observed in cells of *M. lysodeikticus* after inhibition of peptidoglycan synthesis by D-cycloserine (King and Grula, 1972). In cells of *S. aureus* the surrounding cell wall was thinner than normal and the cell wall septa showed a loss in density and gross irregularity in shape after exposition to penicillin (Murray et al., 1959). Klainer and Perkins (1970) observed by scanning beam electron microscopy that exposure of cells of *S. aureus* to relatively low penicillin concentrations resulted in the appearance of small bleb-like structures on the surface of occasional cells and irregular spherical structures lying free or appearing to extrude from the cells. Exposure to higher concentrations caused a more frequent occurrence of these phenomena as well as the occurrence of cells of three morphological, "types": (i) cells with cobblestone surface appearance; (ii) cells exhibiting a mosaic-like surface structure and (iii) large smooth cells. Besides these types of cells also large, irregularly shaped cells occurred. In a strain of *B. licheniformis* penicillin in relatively low concentrations caused uncontrolled cell wall synthesis leading to localized cell wall thickening at the expense of elongation. This resulted in growth in twisted and coiled chains. In higher concentrations cells were produced which had a thinner cell wall than normal cells (Highton and Hobbs, 1971).

None of the effects of inhibition of cell wall synthesis by antibiotics could be observed in *B. bifidum* var. *pennsylvanicus*. We must bear in mind, however, that the cell wall in this organism is relatively thin. This implies that an occurring decrease in wall thickness is difficultly to establish.

b. Omission of N-benzoyl-glucosamine

Inhibition of peptidoglycan synthesis was also studied in cells which were incubated for 3 h in a medium lacking N-benzoyl-glucosamine. During this relatively short incubation time there was no development of bifid forms. Remarkable was the development of "bands" of material near the septum in dividing cells (Fig. 52). These bands remained after cell separation for some time and faded away after prolonged incubation.

The character and significance of these bands is not known. Their development might mean that in dividing cells the lack of peptidoglycan is compensated for by supply of other material. This material will predominantly be deposited in the septum region, since cell division cannot take place without a completed septum. The material will be deposited not only at the location where the cells are in intimate contact, but also in those regions which have already been separated from each other. This causes two bands of material symmetrical to the plane of division. Such rings might be interesting markers in the study of the growth of bacterial cell walls.

SUMMARY

In Gram-positive bacteria the cell envelope consists of two components: cell wall and cytoplasmic membrane. Although they have different structures and functions, both are required for normal growth and function of the organism. There is relatively little known about interactions between cell wall and cell membrane in Gram-positive bacteria. We studied this using the Gram-positive organism *Bifidobacterium bifidum* var. *pennsylvanicus* as a model. Experiments were performed in which either the formation of the membrane or the cell wall was disturbed by antibiotics or growth in media lacking certain nutrients. Several aspects of the formation of cell wall and cell membrane were studied by measuring the incorporation of radioactive precursors, the turnover of membrane and cell wall components, the membrane lipid and protein composition, the protoplast stability, the electrolyte permeability and content of the cells and their ultrastructure.

In Chapter 1 the main aspects of structure, function and biosynthesis of the bacterial cell wall and cell membrane are reviewed. The inhibitory effects of the antibiotics, which have been used in our investigations are described. Chapter 1 also includes a description of the characteristics of the organism *Bifidobacterium bifidum* var. *pennsylvanicus*.

In Chapter 2 the formation of the cell envelope components is studied by measuring the incorporation of [^{3}H]glycine, [^{14}C]oleic acid and N-benzoyl-[^{14}C]glucosamine into the membrane protein, membrane lipids and cell wall peptidoglycan. The most remarkable finding was that the synthesis of peptidoglycan and membrane lipids continued notwithstanding nearly complete inhibition of protein synthesis. Inhibition of peptidoglycan synthesis caused an increased incorporation of oleic acid and palmitic acid into the membrane lipids.

Chapter 3 deals with the turnover of cell wall and cell membrane components, during relatively short incubation periods. No turnover of the peptidoglycan and cellular protein was found during normal growth and during inhibition of protein and peptidoglycan synthesis.

The results suggest that no turnover of membrane protein occurs during normal growth and inhibition of protein synthesis and only a slight turnover during inhibition of cell wall synthesis. A remarkable phenomenon was that during inhibition of peptidoglycan synthesis a significant turnover of oleic acid-labeled membrane lipids occurs accompanied by the secretion of phospholipids and glycolipids. The secreted lipids were the same as those present in the membrane. The secretion of lipids appeared not to be due to lysis or extensive damage of the cells.

Chapter 4 describes changes in the composition and function of the cell membrane. Upon inhibition of protein synthesis the lipid-galactose content of the cells increased significantly, while upon inhibition of peptidoglycan synthesis the lipid-phosphorus content tended to increase. Despite the secretion of glycolipids and phospholipids after cell wall inhibition, the lipid content did not decrease, suggesting that lipids are being replaced or synthesized in excess. Antibiotics inhibiting peptidoglycan synthesis caused significant changes in the fatty acid composition of the polar lipids. These changes may have contributed to the increased incorporation of oleic acid into the membrane, although they are not the main cause.

There were no indications for changes in the amino acid and protein composition of the cell membrane upon inhibition of protein and peptidoglycan synthesis by antibiotics and nutrient depletion. Protoplasts of cells, in which peptidoglycan synthesis was inhibited, appeared to be osmotically more stable than those of normal cells, whereas they were osmotically less stable upon inhibition of protein synthesis. There was a correlation with the sodium and potassium contents of the intact cells. Due mainly to the accumulation of potassium ions during inhibition of protein synthesis and the efflux of potassium ions during inhibition of peptidoglycan synthesis, the internal osmotic pressure of the cells may have been changed. An increased permeability for $^{86}Rb^{+}$ in intact cells was demonstrated upon inhibition of peptidoglycan synthesis, whereas inhibition of protein synthesis caused the reverse effect.

In Chapter 5 the ultrastructure of the cells is studied. The cells

exhibited no signs of lysis or extensive damage upon inhibition of protein and peptidoglycan synthesis. An increased occurrence of mesosomal structures was observed. Upon inhibition of protein synthesis there was no detectable thickening of the cell wall, probably because of the initial thinness of the cell wall. Remarkable was the occurrence of sections in the plasma membrane with a large electron-transparent gap between the electron-dense leaflets. This phenomenon may be related to the increased lipid content of the membrane and caused by local accumulation of lipids.

Inhibition of peptidoglycan synthesis by antibiotics caused no detectable effects on the ultrastructure of the cells. However, However, a relatively short period of incubation in a medium, lacking human milk caused the formation of bands of electron-dense material at the polar sides of the cells. In dividing cells these bands were found symmetrically to the plane of division. After prolonged incubation these bands disappeared and bifurcation occurred.

SAMENVATTING

Gram-positieve bacteriën zijn omgeven door een celwand en een celmembraan. Hoewel deze onderdelen van de cel een verschillende structuur en functie bezitten zijn ze beide nodig voor een normale groei van het organisme. Er is betrekkelijk weinig bekend over de interacties tussen de celwand en celmembraan bij Gram-positieve bacteriën. Om dit te bestuderen werd door ons het Gram-positieve organisme *Bifidobacterium bifidum* var. *pennsylvanicus* als model gebruikt. Het organisme werd blootgesteld aan omstandigheden waarin de vorming van celwand of celmembraan werd verstoord. Onder deze omstandigheden werden verschillende aspecten van de vorming van membraan en celwand bestudeerd, zoals: incorporatie van radioactieve bouwstenen in de celmembraan en de celwand, de turnover van de membraan- en celwandcomponenten, de samenstelling van membraanlipiden en -eiwit de protoplaststabiliteit, de permeabiliteit voor electrolyten, het electrolyt-gehalte van de cellen en hun ultrastructuur.
Hoofdstuk 1 behandelt de structuur, functie en biosynthese van celwand en celmembraan bij bacteriën en de voornaamste eigenschappen van het organisme *B. bifidum* var. *pennsylvanicus*. De werking van een aantal antibiotica die bij onze experimenten zijn gebruikt is uiteengezet.
Hoofdstuk 2 gaat over de inbouw van [^{3}H]glycine en [^{14}C]oliezuur en N-benzoyl-[^{14}C]glucosamine in respectievelijk celmembraan en celwand. Opmerkelijk is dat de incorporatie van bouwstenen in de membraanlipiden en het peptidoglycaan van de celwand doorgaan ondanks een nagenoeg volledig geremde eiwitsynthese. Remming van de celwandsynthese veroorzaakt een verhoogde inbouw van oliezuur en palmitinezuur in de membraanlipiden.
Hoofdstuk 3 behandelt de turnover van celmembraan- en celwandcomponenten gedurende betrekkelijk korte incubatietijden. Tijdens normale groei en remming van de eiwitsynthese wordt geen turnover van het celeiwit en peptidoglycaan waargenomen. Onder deze omstandigheden is er waarschijnlijk geen turnover van het membraaneiwit, terwijl na remming van de celwandsynthese een geringe turnover van het membraaneiwit plaatsvindt. Opmerkelijk is de turnover van met oliezuur gemerkte lipiden na remming van de celwandsynthese. Dit verschijnsel

gaat gepaard met de uitscheiding van glyco- en fosfolipiden. Deze lipiden komen in samenstelling overeen met die van de membraan. Deze uitscheiding is geen gevolg van lyse of ernstige beschadiging van de cellen.

In Hoofdstuk 4 worden veranderingen in de samenstelling en de functie van de celmembraan beschreven. Het lipiden-galactosegehalte van de cellen neemt na remming van de eiwitsynthese toe, terwijl na remming van de celwandsynthese het lipoid-fosforgehalte tendeert naar een toename. Ondanks de uitscheiding van lipiden in het medium tijdens remming van de celwandsynthese neemt het lipiden-galactose- en lipoid-fosforgehalte in de cellen niet af. Dit suggereert dat in de cel de uitgescheiden lipiden worden vervangen of dat deze in overmaat worden gesynthetiseerd. Een aantal antibiotica die de celwandsynthese remmen veroorzaken significante veranderingen in de vetzuursamenstelling van de polaire lipiden. Deze veranderingen zijn echter niet de voornaamste oorzaak van de verhoogde inbouw van oliezuur in de membraanlipiden. Er zijn geen aanwijzingen dat de membraaneiwitsamenstelling verandert na remming van de celwand- en eiwitsynthese. Protoplasten van cellen, waarvan de celwandsynthese is geremd, bezitten een grotere osmotische stabiliteit dan protoplasten van normale cellen, terwijl het tegenovergestelde het geval is bij cellen, waarvan de eiwitsynthese is geremd. Na remming van de celwandsynthese is er bij intacte cellen een verhoogde permeabiliteit voor $^{86}Rb^{+}$ ionen en neemt het kaliumgehalte van de cellen af. Na remming van de eiwitsynthese is er een verhoogde opname van $^{86}Rb^{+}$ ionen en neemt het kaliumgehalte van de cellen toe. De hierdoor veranderde inwendige osmotische druk van de cellen kan mogelijk de oorzaak zijn van het verschil in protoplaststabiliteit.

In Hoofdstuk 5 wordt de ultrastructuur van de cellen behandeld. De cellen vertonen na remming van de eiwit- en celwandsynthese geen tekenen van lyse of ernstige beschadiging. Waarschijnlijk ten gevolge van de oorspronkelijk geringe dikte van de celwand wordt geen verdikking van de celwand na remming van de eiwitsynthese waargenomen. Onder deze omstandigheden komt in sommige gedeelten van het plasmamembraan een betrekkelijk grote ruimte voor tussen de twee electronendichte lagen. Dit verschijnsel staat mogelijk in verband met een

verhoogd lipidengehalte van de membraan en wordt misschien veroorzaakt door een locale ophoping van lipiden. Remming van de celwandsynthese veroorzaakt geen opmerkelijke effecten op de ultrastructuur. Echter, na een korte periode van incubatie in een medium zonder moedermelk onstaan op de polaire gedeelten van de bacterie banden van electronendicht materiaal. In delende cellen bevinden deze zich symmetrisch ten opzichte van het vlak van deling. Na langere incubatieduur verdwijnen de banden en wordt bifurcatie waargenomen.
Na remming van de celwand- en eiwitsynthese worden mesosoom-structuren vaker aangetroffen dan in normale cellen.

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A B B R E V I A T I O N S

Az	: cross-linking amino acid
A260	: absorbance at 260 nm
A550	: absorbance at 550 nm
cpm	: counts per minute
dpm	: desintegrations per minute
GlcNAc	: N-acetyl-D-glucosamine
HM	: human milk
lipid-P	: lipid-phosphorus
lipid-Gal	: lipid-galactose
MurNAc	: N-acetyl-muramic acid
P_i	: inorganic phosphate
PP_i	: inorganic pyrophosphate
SD	: standard deviation
SDS	: sodium dodecylsulphate
TCA	: trichloroacetic acid
Tris	: tris-(hydroxylmethyl)-aminomethane
UV	: ultraviolet

C U R R I C U L U M V I T A E

De schrijver van dit proefschrift behaalde zijn einddiploma H.B.S.-B aan het St. Jacobuscollege te Enschede in 1964. In datzelfde jaar begon hij zijn scheikunde studie aan de Katholieke Universiteit te Nijmegen. Hij behaalde zijn doctoraalexamen (hoofdvak Biochemie) in december 1970 (cum laude). Daarna was hij als wetenschappelijk medewerker verbonden aan de Medische Faculteit te Nijmegen en werkzaam op het Centraal Isotopen Laboratorium van de afdeling Biochemie. Sinds 1 september 1974 is hij in dienst van het Staatstoezicht op de Volksgezondheid, als inspecteur van de Volksgezondheid binnen de Inspectie voor de hygiëne van het milieu in de provincie Zuid-Holland.

STELLINGEN

I

Indien voor de vorming van protoplasten uit Gram-positieve bacterien gebruik wordt gemaakt van penicilline, dient rekening te worden gehouden met veranderingen in de lipidensamenstelling en de permeabiliteit van de membraan

Dit proefschrift, Hoofdstuk IV.

II

Montgomerie *et al.* houden bij hun veronderstelling, dat de osmoregulatie van *Streptococcus faecalis* plaats vindt door veranderingen in het vetzuurpatroon, geen rekening met eventuele andere wijzigingen in de lipidensamenstelling.

J.Z. Montgomerie, G.M Kalmanson en L.B. Guze
J. Bacteriol. 115(1973)73

III

De door Fujino *et al.* gegeven waarden, die een enzymatische synthese van sphingomyeline uit ceramide en CDP-choline moeten adstrueren, kunnen niet als significant worden beschouwd.

Y Fujino, T Negishi en S. Ito
Biochem J. 109(1968)310

IV

De door Prasad *et al.* aangetoonde inbouw van radioactieve aminozuren in lipidenfracties van rattenlever bewijst geenszins dat deze aminozuren worden gebruikt voor de synthese van aminolipiden.

R Prasad, N K Garg en C R. Krishna Murti
Indian J. Biochem.Biophys. 9(1972)185

V

De resultaten van Heisler *et al.* met betrekking tot de afgifte van spijsverteringsenzymen vanuit pancreasweefsel zijn disputabel

S Heisler, G Grondin en G. Forget
Life Sci. 14(1974)631

VI

Daar de eigenschappen van chloroform-methanol mengsels als extractiemiddel voor lipiden in biologische materialen niet uniek zijn, is het mogelijk deze mengsels te vervangen door gelijkwaardige systemen met een grotere chemische stabiliteit en lagere toxiciteit

P. Schmid, J Calvert en R. Steiner
Physiol Chem. & Phys 5(1973)157

VII

In het kankeronderzoek dient meer aandacht besteed te worden aan een mogelijke rol van producten van de darmflora bij de carcinogenese.

B S. Drasar en M.J. Hill
Am J.Clin.Nutr. 25(1972)1399

VIII

Het is onjuist te veronderstellen dat alle biologisch afbreekbare stoffen onschadelijk voor het milieu zouden zijn

IX

Het is onnodig dat tandartsen het teken van de zwarte esculaap op hun auto's voeren, daar het zelden voorkomt dat zij zich naar hun patienten spoeden.

G C Molenkamp 20 juni 1975